EXPLORING THE POSSIBILITIES OF ENHANCING ENZYMATIC ACTIVITIES OF PHYTOPATHOGENIC FUNGI FOR THEIR UTILITY IN BIOLOGICAL CONTROL OF WATERHYACINTH

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I. ABSTRACT

Several phytopathogenic fungi often cause damage to their host-plants' tissue, producing metabolites which induce disease symptoms (such as chlorosis, necrosis, etc.) that eventually lead to the death of the plants and in case of weeds, their control. Among the several phytopathogens (identified by 18S rDNA techniques), collected from and host-specific to waterhyacinth (*Eichhornia crassipes*), few have shown to produce an array of lytic, cell-wall-degrading enzymes (CWDEs) that can degrade the polysaccharides of plant cell walls and break the structural moiety of the plants. On the basis of the damage intensity incurred by the primary metabolites on the host weed, the present study highlights on one of the initial reports, where xylanases (in its optimised condition) produced by *Fusarium oxysporum* sp. lycopersici 4287, have shown potency against its targeted host, waterhyacinth (a noxious aquatic weed) by aiming the xylans, which are a major hemicellulosic component of the cell walls. This eventually helps to break the structural rigidity and moiety of the plant and leading to control its invasiveness.

Keywords: *Eichhornia crassipes*, *Fusarium oxysporum* f. sp. *lycopersici* 4287, primary metabolite, CWDEs, biocontrol, xylanase

II. INTRODUCTION

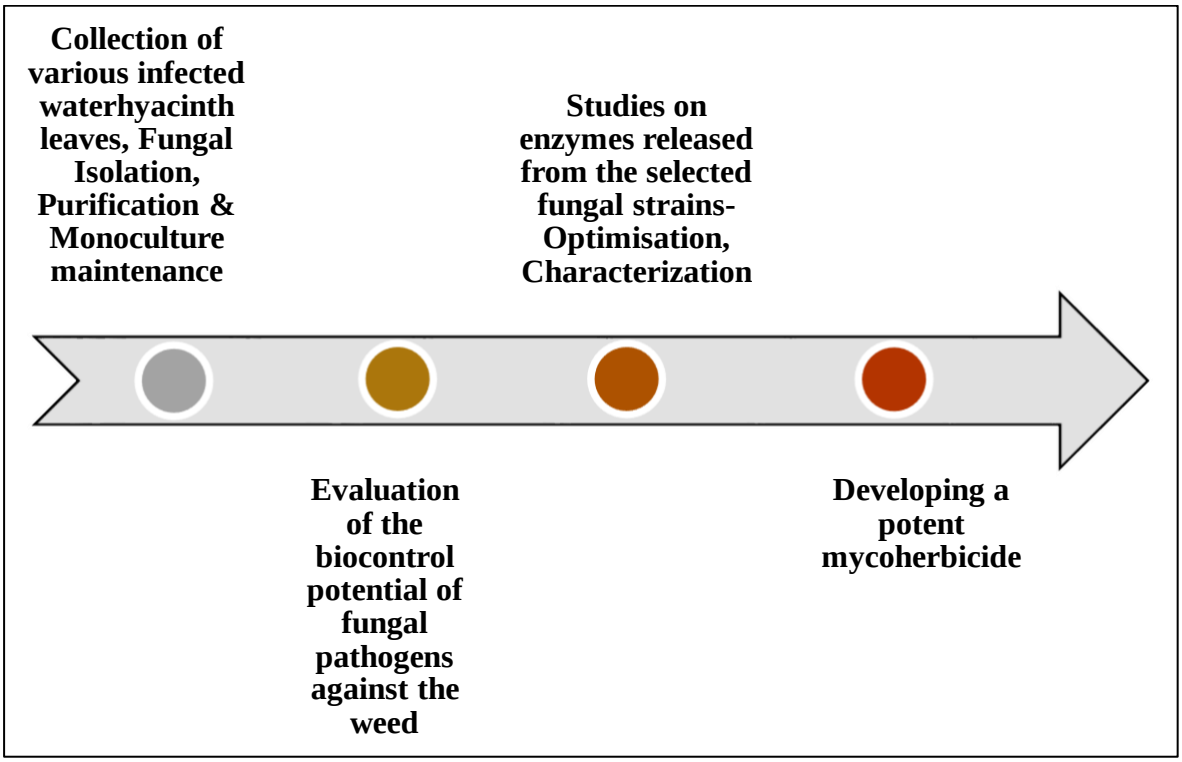
The aquatic weed, waterhyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), is considered as one of the ten worst weeds of the world (Asmare et al., 2020, Holm et al., 1977). Waterhyacinth is well known for its negative impact on economy and ecosystem worldwide (Ray and Hill, 2013a). Several control mechanisms in the form of manual, mechanical, chemical and biological methods have been executed for controlling the invasiveness of waterhyacinth with varying results (Charudattan et al, 1996). However, various environmental and financial challenges are associated with these methods. Over last few decades, lot of emphasis has been on adapting eco-friendly measures, were several biocontrol agents including arthropods and plant pathogens have been released around the world to control the weed (Ray and Hill, 2013a; Firehun et al, 2015). Biocontrol agents have often been found to be slow and unpredictable (Ray and Hill, 2016). Hence, more efforts are required to improve their biocontrol efficacy. Studies show that pathogens can cause significant reduction in waterhyacinth biomass, especially following insect attack or natural disease outbreak (Ray and Hill, 2013b). Several studies on isolation, identification and biocontrol potential of the fungi have been tested in various infested areas worldwide (Daddy et al. 2003, Freeman et al. 1981, Naseema and Balakrishnan 2001, Okunowo et al. 2008, Praveena and Naseema 2006, Ray 2008). However, the continual survey and lookout of several new species can reduce the stress on limited species and broaden the list for agents against this weed, apart from the some of the most commonly used biocontrol agents like various species of *Alternaria, Acremonium, Fusarium* etc. Pathogens, which are also native or endemic, are comparatively found to be more potent and ideal for the

development of biocontrol agents (Cuda et al. 2008). Studies involving interaction with other biocontrol agents apart from phytopathogens gives a glimpse into a reality, rather bigger picture, of the surrounding environment (Dutta and Ray, 2017). Plant biomass (Brower and Zar, 1984) is mostly made up of polysaccharides. Various fungus acts on these polysaccharides to produce their respective enzymes (Ismail, 1995). Okunowo et al., (2010) studied cellulolytic and xylanolytic activity of a pathogenic *Myrothecium roridum* Tode ex Fr., non-pathogenic *Fusarium solani* (Mart.) Sacc. and *Curvularia pallescens* Boedijn isolates from waterhyacinth. Of the three isolates, *M. roridum* is capable of producing cellulases and xylanase enzyme in submerged cultures but to a lesser degree compared to *F. solani* and *C. pallescence* (Okunowo et al., 2010). Fungi are known to release primary metabolites, often enzymatic in nature and capable of degrading/damaging plant cell wall, known as cell-wall degrading enzymes (CWDEs). Several genes have been obtained from phytopathogens and it was determined that most genes play an important role in pathogenicity (Chen et al. 2006). Although lot of studies has been done using the fungi as control agent of the weed, yet no work has been done to study their primary metabolites (which embarks the breakdown of the plant cell wall during initiation of disease) as bioherbicides. Enzymatic degradation of plant polysaccharides has been used in fuel and many other industries but no such literature of CWDEs associated with weed biocontrol, have been reported. Hence aiming to focus on the potentiality of such metabolites against this weed, the possibilities of studying the enzymes and enhancing their activity through physiochemical studies to improve their bioherbicidal potential against waterhyacinth, was studied.

III. OBJECTIVES:

1) Survey for collection of diseased leaves and isolation, purification and identification of fungal strains associated with waterhyacinth
2) Evaluation of the biocontrol potential of fungal pathogens against the weed
3) Studies on enzymes released from the selected fungal strains
4) Optimizing, characterizing and exploring possibilities of application of the isolated enzymes with emphasis on enhancing waterhyacinth biocontrol.

PLAN OF WORK:


Collection of various infected waterhyacinth leaves, Fungal Isolation, Purification & Monoculture maintenance
Studies on enzymes released from the selected fungal strains- Optimisation, Characterization
Evaluation of the biocontrol potential of fungal pathogens against the weed
Developing a potent mycoherbicide

IV. MATERIALS AND METHODS

1. OBJECTIVE 1: *Survey for collection of diseased leaves and isolation, purification and identification of fungal strains from waterhyacinth*

Periodical surveys were conducted to various waterhyacinth-infested sites and disease symptoms bearing leaves, suspected mostly to be damaged, by fungal pathogens were collected and brought to laboratory, and were thoroughly washed in running tap-water to remove unrequired soil particles. $2mm^2$ cross-sectional segments from the margins of necrotic and chlorotic lesions of the infected leaves and petioles were taken and surface sterilized with sodium hypochlorite (NaOCl) solution. The sections were inoculated on Potato Dextrose Agar (PDA) media plates and incubated, in Biological Oxygen Demand (BOD) incubator, at 27^oC. The fungal colonies were purified by sub-culturing techniques (Agarwal and Hasija 1986) until pure monocultures were obtained. The purified isolates were numbered and multiplied on PDA plates and maintained at 4^oC refrigeration for future use.

The fungal strain, isolated, was morphologically identified by slide cellophane (Butler and Mann, 1959), slide culture technique (Harris 1986) with Lactophenol as mounting medium, and observed under Zeiss Axio Scope.A1 Microscope for morphological identification of the genus using pictorial book references (Agarwal and Hasija 1986, Barnett 1960, Holliday 1993, Robinson 2011, Watanabe 2010). The conidial surfaces obtained from cultures were later analysed for scanning

electron microscopy (SEM) following Melo and Faull's method (Melo and Faull, 2004). Confirmation of morphologically identified fungal species were done by molecular characterisation using 18S rDNA molecular technique, followed by BLAST of the consensus sequences, with nr database of NCBI GenBank database and phylogenetic analysis.

(Primer used: 1F: CTGGTGCCAGCAGCCGCGGYAA
4R: CKRAGGGCATYACWGACCTGTTAT)

Frequency of occurrence of fungal isolates

The frequency of appearance of fungi, isolated from various waterhyacinth-infested sites, were counted with respect to the other genera. The frequency of each genus was expressed as percentage of the total number of fungal isolates, using the formula (Ray and Hill 2013a):

$$Frequency\ (\%) = \frac{Number\ of\ isolates\ in\ a\ genus\ x\ 100}{Total\ no.\ of\ isolates}$$

The experimental data were analysed using SPSS version 25. The frequency fungal isolates data were analysed using Chi square test.

2. OBJECTIVE 2: *Evaluation of the biocontrol potential of fungal pathogen against the weed*

Test plant preparation

The plants, used in this study, were collected from waterhyacinth-infested waterbodies, at their early growth stage. They were brought back, grown and maintained in big plastic/ cement tanks, as stock culture, at Presidency University, Kolkata, India. The plants were supplemented with farm yard manure and 15-3-12 N:P:K, slow-release fertilizer. For experimentation, plants were kept in the growth chamber conditions at 26^{o}C temperature and 75 to 85% relative humidity (RH) in plastic pots (18mm diameter) filled with fertilized water.

Fungal mycelial and spore suspension preparation

A 14 days old culture of the test fungi, grown in 90mm PDA plates, were scraped out, by flooding the Petri plates with sterile distilled water. A sterile spatula was used to obtain a fungal suspension containing 10g of scraped material per 50 ml of sterile distilled water.

To understand the disease-causing potential of the fungi, the isolates were tested for their infection causing ability against waterhyacinth plants in vitro, initially by detached leaf bioassay followed by whole plant bioassay, under laboratory and greenhouse conditions, using fungal mycelium and spore suspension (10^5 - 10^7 spores/ml), measured using haemocytometer counting technique (Singh and Singh

2007), along with Tween 20 as surfactant (Ray 2008). The presence of disease, was determined in terms of the percentage of number and area of the plant leaves that exhibited disease symptoms. On the basis of these visual observations, disease intensities recorded and categorized as excellent (+++), good (++), poor (+), and no infection (−) on virtue of a pictorial disease scale (Freeman and Charudattan, 1984; Dutta and Ray, 2017). Disease was scored using a 0-9 scale rating system where 0 signified healthy and 9 indicated 90% diseased area covered by necrosis or chlorosis on leaves, until the death or damage of the entire plant or almost all the plants. The fungi showing good potential were used for further studies.

The quantitative numerical values are presented with mean and SEM. One way analysis of variance (ANOVA) was used to compare the disease intensity mean values of different isolates tested at each hours of exposure. A repeated measure of ANOVA was used to compare the means of disease density values at 24, 48, 72, 96, and 120 hrs of exposure.

Host-range specificity test

Ecologically and economically important plants, related to waterhyacinth, were used to check the specificity of the various fungal pathogens on them, in order to understand the efficacy of these pathogens, prior to their application for the biocontrol of the host weed on its various infested sites, Four potential fungal biocontrol agents of waterhyacinth were subjected to host range studies. Experimental inoculations of 67 plant species belonging to 30 families with mycelial suspension and crude metabolite of the respective fungi was done. For the host-range, study inoculum was harvested by flooding these colonies with sterile water, disrupting the colony surfaces by scraping with the edge of a flamed microscope slide and filtering the resulting suspension through two layers of cheesecloth. Mycelial suspension was prepared using 10 mg of mycelial mat in 50 ml sterile distilled water while fungal crude metabolite was prepared using 21-day-old metabolised broth filtered from actively growing culture of the fungus. Conidial density of the mycelial suspension was measured using haemocytometer and it was adjusted to the required density by dilution with sterile water. For each plant species tested, two sets of test plants were prepared. Each set contained five pots and each pot contained between two and five plants. Test plants were propagated either from seed, cuttings or obtained from nursery. The test plants were inoculated when they had 3–5 fully mature leaves.

Arthropod- plant-pathogen interaction studies

A study was done in order to understand the mutual impact of different biocontrol agents of waterhyacinth, among themselves, as well as on the biocontrol of the target weed. Fungal strains, having severe disease-causing potential were selected for this study and applied on waterhyacinth, singly and in different combinations with the arthropod biocontrol agents of the weed, including the chevroned waterhyacinth weevil or *Neochetina bruchi* Hustache (Curculionidae:Coleoptera)

(NB), and waterhyacinth mite or *Orthogalumna terebrantis* Wallwork (Galumnidae :Acarina) (OT).

The data were subjected to analysis of variance (ANOVA) using statistical programme Statistica. The means of the treatment were compared with least significant difference (LSD) at 5% level of significance.

3. OBJECTIVE 3: *Studies on enzymes released from the selected fungal strains*

The potential strain/s were cultivated in Potato Dextrose Broth (PDB) were inoculated in liquid media with different carbon source (pure carboxymethyl cellulose, cellobiose, xylan, pectin), with an ambient cultivation condition of pH 7 for 48 hours at 27^{o}C, to obtain their respective enzymes. The fermented culture media, bearing the enzyme, in their crude form, were then centrifuged at 10,000 rpm for 10 min and the supernatant used as the crude enzyme. A part of the crude enzyme was used to check the activity of each type of enzyme and part to check the biocontrol potential and pathogenicity of the enzymes produced, against waterhyacinth. The disease intensity was measured, based on a pictorial disease scale (Freeman and Charudattan, 1984), as mentioned previously.

To understand the activity of the enzyme, the assay mixture (1 ml) containing an equal volume of enzyme and 1% (w/v) of respective substrate dissolved in 0.1 (M) phosphate buffer (pH 7) incubated at 37°C for 10 min, was measured. The reducing sugar released in each case, was measured by the dinitrosalicylic acid (DNSA) method **(**Miller, 1959) and the absorbance of the enzymes was correlated using a standard (glucose) product curve to quantify the concentration (in Enzyme Unit/ml or U/ml).

4. OBJECTIVE 4: *Optimizing, characterizing and exploring possibilities of application of the isolated enzymes with emphasis on enhancing waterhyacinth biocontrol*

The fungi, which produced potent enzymes showing pathogenicity and enzyme activity were selected for optimisation and characterization. The concentrations of different substrates used as sole carbon source, was varied from 0.5% - 3% to optimize their concentration in submerged culture of the specific fungus isolated. The most favourable production temperature, optimum pH, and time course of growth and enzyme production by the selected strain under optimized culture conditions for enzyme production was determined, in order to understand the effective active conditions of the primary metabolites.

The supernatant was subjected to fractionated ammonium sulphate (45-60% w/v) precipitation followed by centrifugation (10,000 rpm for 10mins at 4°C) for partial

purification of the enzyme. The pellet was re-suspended in 20 mM Tris HCl-100mM NaCl buffer (pH 8) and the suspension was then dialyzed against the same buffer with PD10 gel-filtration chromatography (pre-packed with Sephadex G25) column, for desalting (Aygan et al. 2008). The salt-free, purified enzyme was spectrophotometrically observed at 290nm to estimate the presence of protein.

Biocontrol potential of the enzymes and their observation at tissue level

Initially all the partially purified enzymes (the activity was measured by DNSA method), in their optimised condition, were applied on fresh untreated waterhyacinth to understand the biocontrol potential and its pathogenicity against waterhyacinth was recorded, based on a pictorial disease scale (Freeman and Charudattan, 1984).

The potent enzyme, among the many, were further analysed by observing their action, at regular interval (after application of the enzyme), on the transverse tissue section of pre-stained untreated waterhyacinth leaves, under microscope (Nikon Trinocular Research Model E with Nikon DSLR model 5100) and images of change were recorded accordingly.

Confirmation of the presence of active genes of the potent fungal enzyme by expression profiling

Substrate-confined fungi inoculated and substrate-less fungi inoculated mat were both collected, weighed and cryopreserved at -80^{o}C, adding liquid N_2. RNA was isolated, from the cryopreserved samples, following the protocol provided by Nucleospin® RNA Plant and Fungi Kit and cDNA prepared using BioRad iScript cDNA Synthesis Kit (1708890) respectively.

The GenBank accession number for glycoside hydrolases family (here, GH9, GH10, GH11), confirming the presence of the FO (*Fusarium oxysporum* sp. *lycopersici* 4289), of the potent enzymes (here, xylanases 2, xylanase 3 and xylanase 5) were recorded and from the FASTA sequences respective primers were designed using Primer 3. With the different sets of primer expression profile of both the substrate-treated and untreated cDNA was analysed to confirm the presence of active gene component of the carbohydrate active enzyme responsible for the cell-wall degradation.

Primer used for:

Xylanase 2 (XYL2)	F: GCTCAATCGGACAAACCAAT
	R: GTCGGTGATGGTTCCGTAGT
Xylanase 3 (XYL3)	F: ATCTTCGCTGAGGACGGTAA
	R: GGCAAGCGTTCATGACAGTA
Xylanase 5 (XYL5)	F: TGGTAAGGGTTGGTCTCCTG
	R: GCTGGAAGGTCTGAGTACCG

Phylogenetic analysis of the active genes of the potential fungal enzyme

Sequence analysis, amino acid alignment and phylogenetic tree analysis for the carbohydrate active enzyme, of the selected fungal species, were carried out using CLC sequence viewer software version 8.0 (Qiagen, Germantown, MD, USA). This was done to understand the relatedness among the different enzymes produced from different species of the selected fungi, grouped into the several categories of the active genes responsible for the cell-wall degradation.

V. RESULTS

1. OBJECTIVE 1: *Survey for collection of diseased leaves and isolation, purification and identification of fungal strains from waterhyacinth*

Samples of diseased waterhyacinth leaves were collected from the field (Figure 1), covering many regions in and around Kolkata and few other regions of West Bengal, India (Figure 2, Table 1).

Variable diseased lesions in the form of leaf spots and/or blights, necrotic flecks, petiole rot, varying sizes and shapes of zonate lesions and dieback symptoms (Figure 3) were brought back to lab for isolating and maintaining the fungi in their pure form (Figure 4).

The 152 isolates belonging to more than 25 genera were purified from diseased plant parts and many eliminated from further consideration because they were either failed to grow, contaminated, or belonged to the non-pathogenic and/or non-sporulating sterile group. Apart from that, some rapid growing isolates on PDA plates, mostly those of *Aspergillus*, *Penicillium* and *Rhizopus*, were excluded from further consideration.

Morphological and molecular analysis grouped the non-eliminated phytopathogens into 17 genera. Many phytopathogens are reported for the first time, against waterhyacinth from the survey. *Fusarium* sp. (Chi square value 38.889 and $P<0.05$) were highly prevalent following the large number of sterile and unidentified fungi. *Fusarium* Link (8.45%), followed by *Alternaria* Nees (6.34%) were the most common genus, found among the potent phytopathogens, isolated from the infected waterhyacinth. Among the 12 isolates of *Fusarium* Link., *F. oxysporum* appeared mostly with 2.11% rate of occurrence followed by *F. verticilloides* (1.41%), *F. roseum*, and *F. ventricosum* both with 0.7% of occurrence. On the other hand, *A. alternata* was prevalent among the *Alternaria* sps., with 4.23% rate of occurrence (Figure 5). *Curvularia* sp. and *Clodosporium* sp. followed with 4.23% and 3.52% rate of occurrence respectively.

Figure 1: Waterhyacinth infestation at various water bodies in and around Kolkata

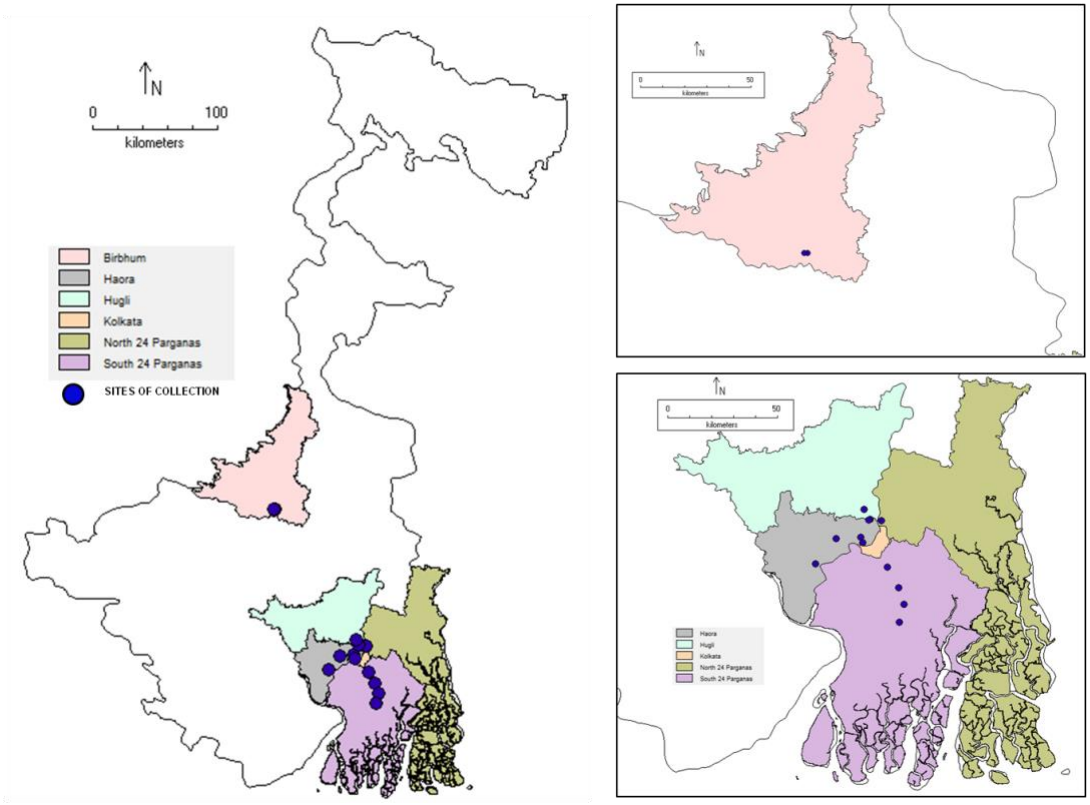
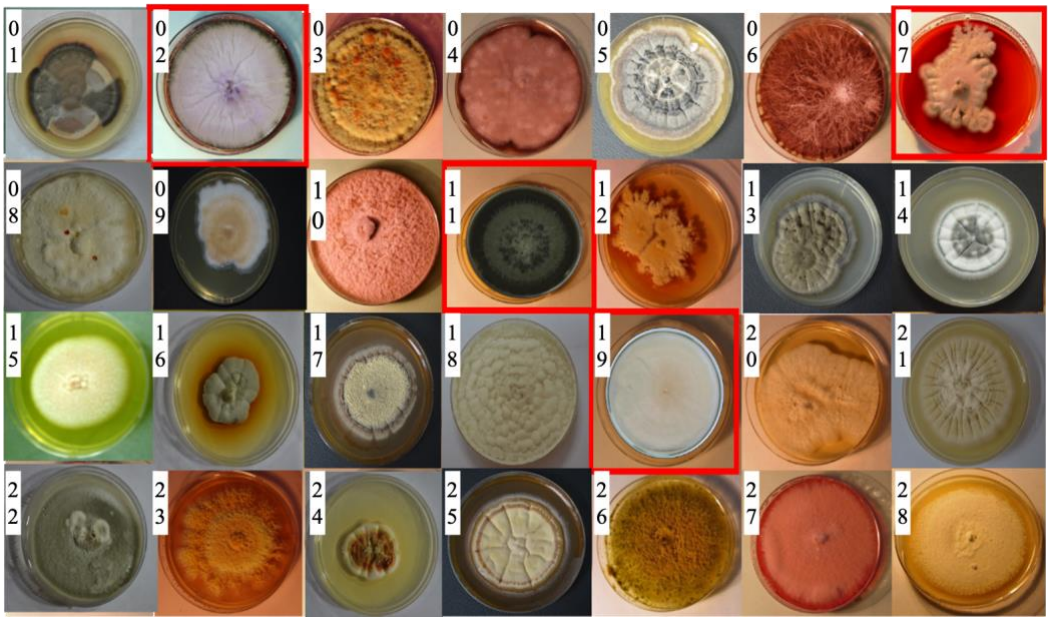


Figure 2: Sites of collection of infected waterhyacinth leaves

TABLE 1: Waterhyacinth infested sites surveyed in 2014–2019 for the collection of the fungi

SL. NO	SITE	LATITUDE (° N)	LONGITUDE (° E)
1	Kandua	22.574627	88.182184
2	Santragachi	22.581372	88.282796
3	Nischinda, Bally	22.655332	88.323845
4	10 No. Rajabagan	22.652371	88.370132
5	Notunpara, Baruipur	22.371033	88.442811
6	Surjopurhat, Baruipur	22.301128	88.464596
7	Dakshin Barasat, S-24 Pgs	22.225514	88.445575
8	Botanical Garden, Shibpur	22.558687	88.291121
9	Rajchandrapur, Bally	22.655029	88.317249
10	Par Dankuni, Hoogly	22.697702	88.296937
11	Uluberia	22.471215	88.094221
12	Bikrampur	22.457071	88.396385
13	Kacharpatty, Santiniketan	23.660825	87.702449
14	Suripukur, Santiniketan	23.66152	87.687244

Figure 3: Collection of various infected leaves from waterhyacinth infested sites



Sl no.	Isolate no.	Fungal isolate
01	*WHK-28*	*Curvularia* sp.
02	*WHK-59*	*Fusarium oxysporum f.* sp. *lycopersici*
03	*WHK-99*	*Fusarium* sp.
04	*WHK-46*	*Alternaria* sp.
05	*WHK-138*	*Pleospora herbarum* (Pers.) Rabenh.
06	*WHK-97*	*Pleospora herbarum* (Pers.) Rabenh.
07	*WHK-3*	*Alternaria alternata* (Fr.) Keissler
08	*WHK-106*	*Fusarium oxysporum f* sp. *cumini* strain F11
09	*WHK-104*	*Arthrinium hispanicum* Larrondo & Calvo
10	*WHK-42*	*Alternaria alternata* (Fr.) Keissler
11	*WHK-37*	*Paradendryphiella salina* (G.K. Sutherland) Woudenberg & Crous
12	*WHK-141*	*Alternaria alternata* (Fr.) Keissler
13	*WHK-152*	*Stemphylium* sp.
14	*Sterile fungi*	*Pencillium sp.*
15	*WHK-17*	*Acremonium* sp.
16	*WHK-38*	*Alternaria alternata* (Fr.) Keissler
17	*WHK-88*	*Fusarium* sp.
18	*WHK-30*	*Myrothecium* sp.
19	*WHK-74*	*Fusarium verticillioides* (Sacc.) Nirenberg
20	*WHK-89*	*Cercospora* sp.
21	*Sterile fungi*	*Aspergillus* sp.
22	*WHK-142*	*Nigrospora* sp.
23	*WHK-19*	*Trichothecium* sp
24	*WHK-96*[#]	*Setosphaeria rostrata* K.J. Leonard
25	*WHK-20*	*Rhizoctonia* sp.
26	*WHK-23*	*Cochliobolus geniculatus* R.R. Nelson
27	*WHK-43*	*Alternaria alternata* (Fr.) Keissler
28	*WHK-12*	*Alternaria japonica* Yoshii 1941

Figure 4: Pure cultures of some fungal flora isolated from waterhyacinth

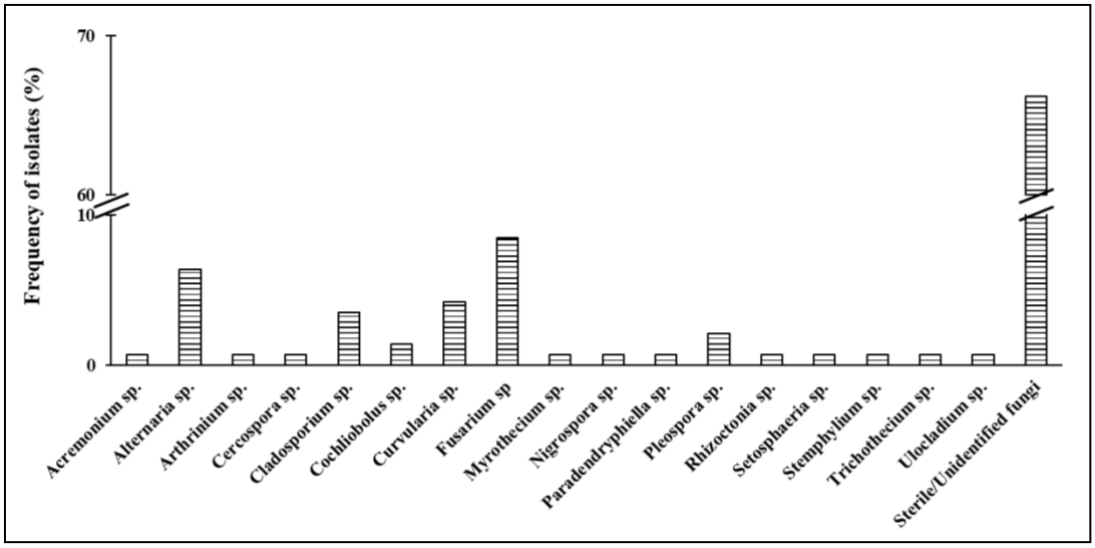


Figure 5: Frequency of occurrence of different fungal genera isolated from infected waterhyacinth from various regions in and out of Kolkata and some parts of Birbhum from 2014–2018

2. OBJECTIVE 2: *Evaluation of the biocontrol potential of fungal pathogen against the weed*

Among the several isolates, the pathogens that caused effective disease to waterhyacinth leaf pieces in pathogenicity testing, performed by detached leaf bioassay, were screened further for their damage (Table 2) and pathogenicity to waterhyacinth by whole plant bioassay (Figure 6). A significant difference in the values of disease intensity in different hrs of exposure of isolates of fungal inoculum on the waterhyacinth leaves (F, 3404, $P<0.05$) was observed (Table 3).

Several *Alternaria* spp, and *Fusarium* spp. were found to be highly effective fungi but apart from them seven effective isolates are reported for the first time from waterhyacinth (Table 4). Based on the disease initiation and intensity of infection, four fungi (Figure 7), *Alternaria alternata* (AA), *Fusarium oxysporum* f. sp. *lycopersici* 4287 (FO), *Fusarium verticilloides* (FR), *Paradendryphiella salina* (PS), were selected for the next round of experiment for host-specificity, arthropod-pathogen interactive study and understanding their nature (Table 5). AA and FO showed maximum pathogenicity among the four intense phytopathogens.

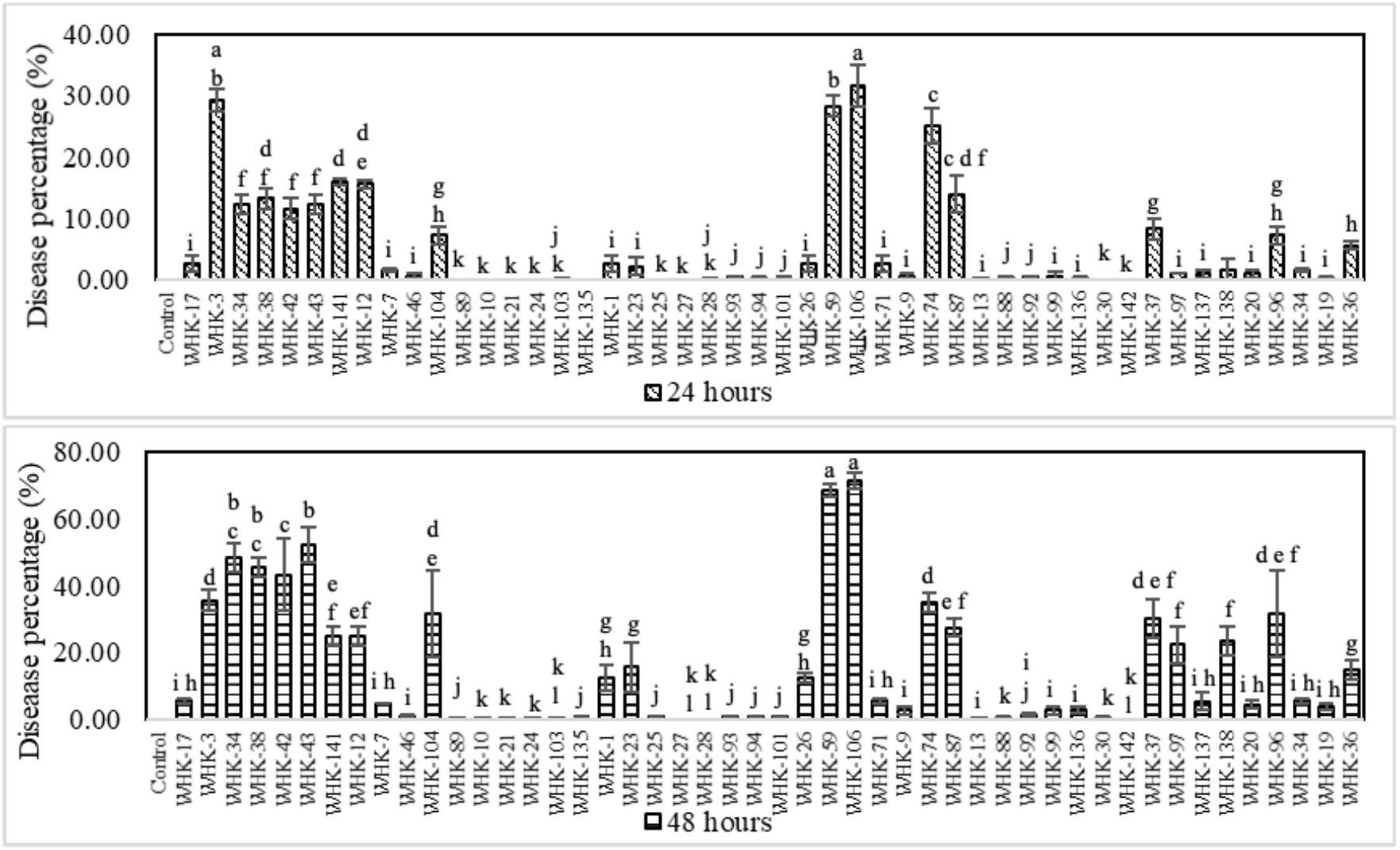
Host-range specificity test

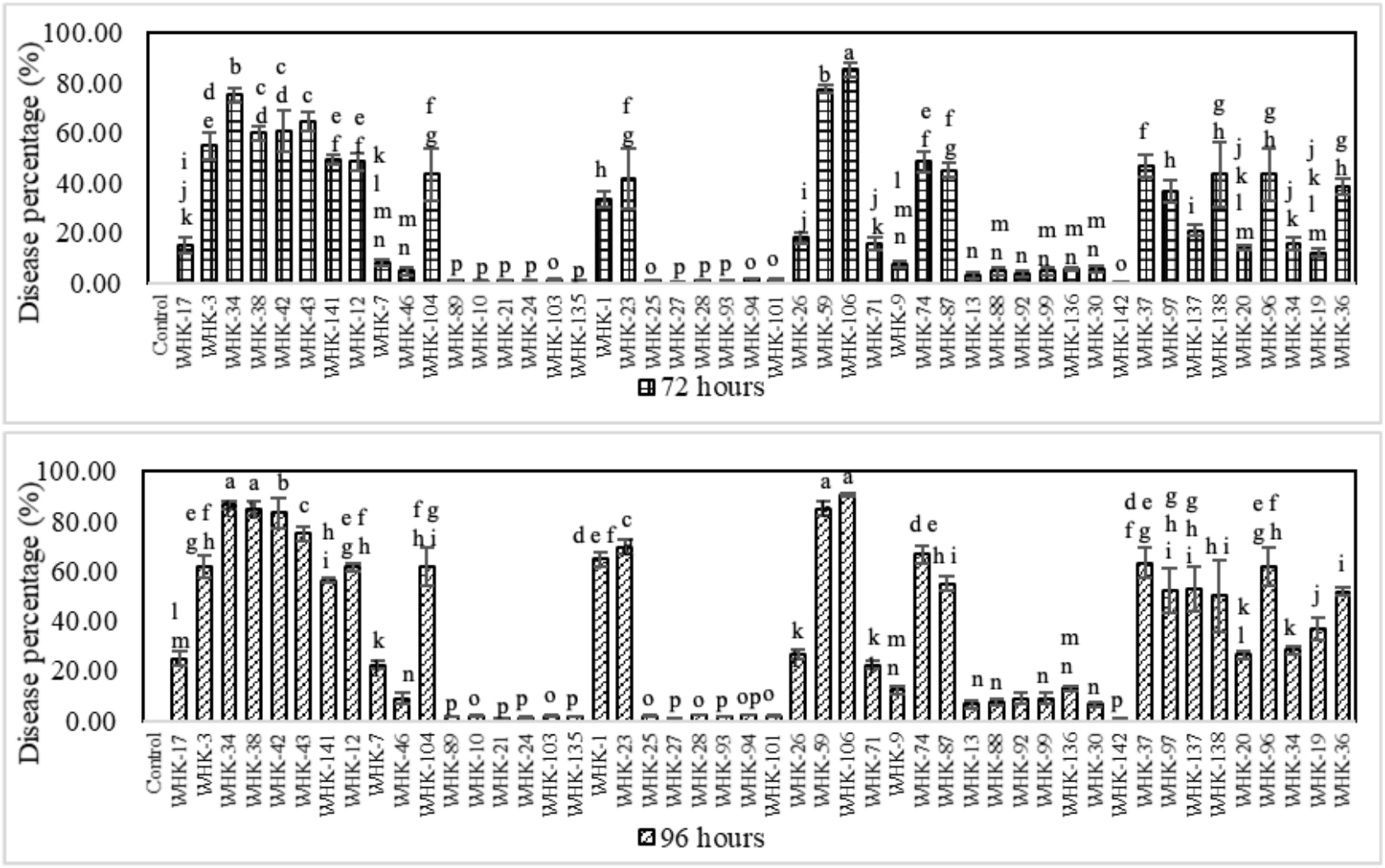
The above-mentioned four potent fungal isolates including AA, FO, FR and PS were selected for host range studies (Figure 8, Table 6). The spore suspension and culture filtrate of the four species were effective against their host, waterhyacinth.

Apart from that, the culture filtrate of AA showed its effect mostly on other weeds like *Pistia stratiotes, Alternanthera sessilis, Trianthema portulascastrum, Sinapis alba, Ipomoea aquatic, Hydrilla verticillata* and *Rumex obtusifolius*, and two cultivated crops like *Spinacia oleracea* and *Chenopodium album*. The spore suspension of AA showed little effects on *C. album*, apart from the host itself. PS had shown its effect at a much broader spectrum, effecting almost all the plants that AA infected and apart from them a few more. In the additional list of infected plants *A. philoxeroides, Amaranthus spinosus, Blumea lacera, Tridax procumbens, Ceratophyllum demersum* all are weeds while *Lycopersicon esculentum* is a cultivated plant. *A. philoxeroides, H. verticillata* and *P. stratiotes* are ecologically related to the host, waterhyacinth. When the culture filtrate is so effective and broad-ranged, the spore suspension of PS is highly host-specific. FO has shown its minimum effect on the two cultivated plants (*S. oleracea* and *C. album*) and a weed (*R. obtusifolius*). The spore suspension of FR has been limited to two aquatic weeds-waterhyacinth and *A. philoxeroides,* while the culture filtrate has shown effects on three out of four *Alternanthera* sps., used for testing, apart from another weed, *Trianthema portulacastrum.*

Arthropod-plant-pathogen interaction studies

Biocontrol agents in various combination had cumulative impact on waterhyacinth. In the arthropod-plant-pathogen interaction study (Figure 9), initially within 24 hours, 13% ± 3.9 damage appeared on waterhyacinth treated with FO and 81.3% ± 5.2 damage within 120 hours. Delayed disease-initiation with 4.0% ± 0.4 necrosis was observed in FO+OT treated plant while FO+NB had a comparatively high damage with 15.0% after 24 hours ($F_{14,75}$=6.67, P=0.00), and enhanced damage percentage of about 89.80% ± 2.8, by 120 hours. PS and AA treated plants showed disease initiation only after 72 hours ($F_{14,75}$=19.30, P=0.00). PS had a repulsive effect with weevils (NB) causing 55.8% ± 4.7 damage after 120 hours ($F_{14, 75}$=22.13, P=0.00), with weevils' death and delayed disease initiation. The combined effect of PS+OT was 23%, a little lower than individual effect of PS (23.7%), and PS+NB interaction resulted even lower damage of 12.7%, after 72 hours. Damage percentage was higher for FR+OT treatment with 94.3% ± 1.2 as compared to FR alone (63.3% ± 12.2), completely opposite to the effect for FR+NB with 32.7%, after 120 hours. Though AA+NB showed a rise within 48 hours of treatment, it was found that AA+OT had had a higher impact on damage causing in the end, causing 89.75 ± 1.13 at the end of 120 hours, while AA and AA+OT showed about 50% damage.


Disease percentage (%)
40.00
30.00
20.00
10.00
0.00
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-59
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
WHK-37
WHK-97
WHK-137
WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36
24 hours
Diseaase percentage (%)
80.00
60.00
40.00
20.00
0.00
48 hours


Disease percentage (%)
100.00
80.00
60.00
40.00
20.00
0.00
72 hours
96 hours
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-99
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
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WHK-97
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WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36

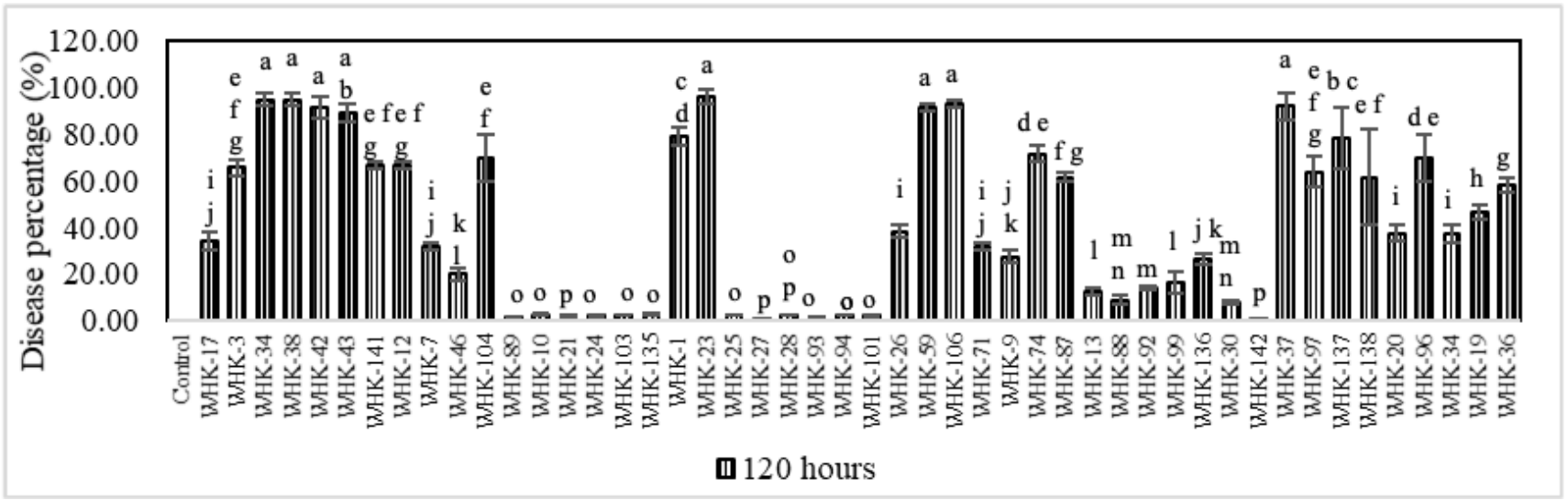


Figure 6: Pathogenicity test of various fungi on waterhyacinth. Mean values marked with different superscripts are significantly different ($P<0.05$).

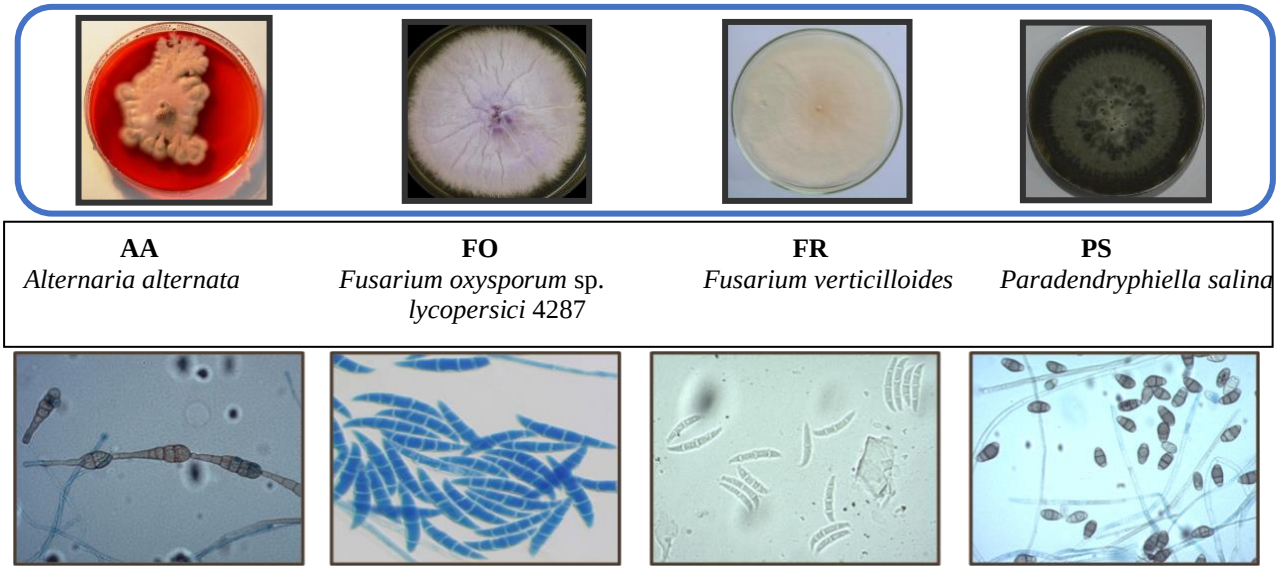


Figure 7: The four selected fungi based on disease initiation and intensity of infection

Figure 8: Host-range pathogenicity trials on several ecologically and economically important plants

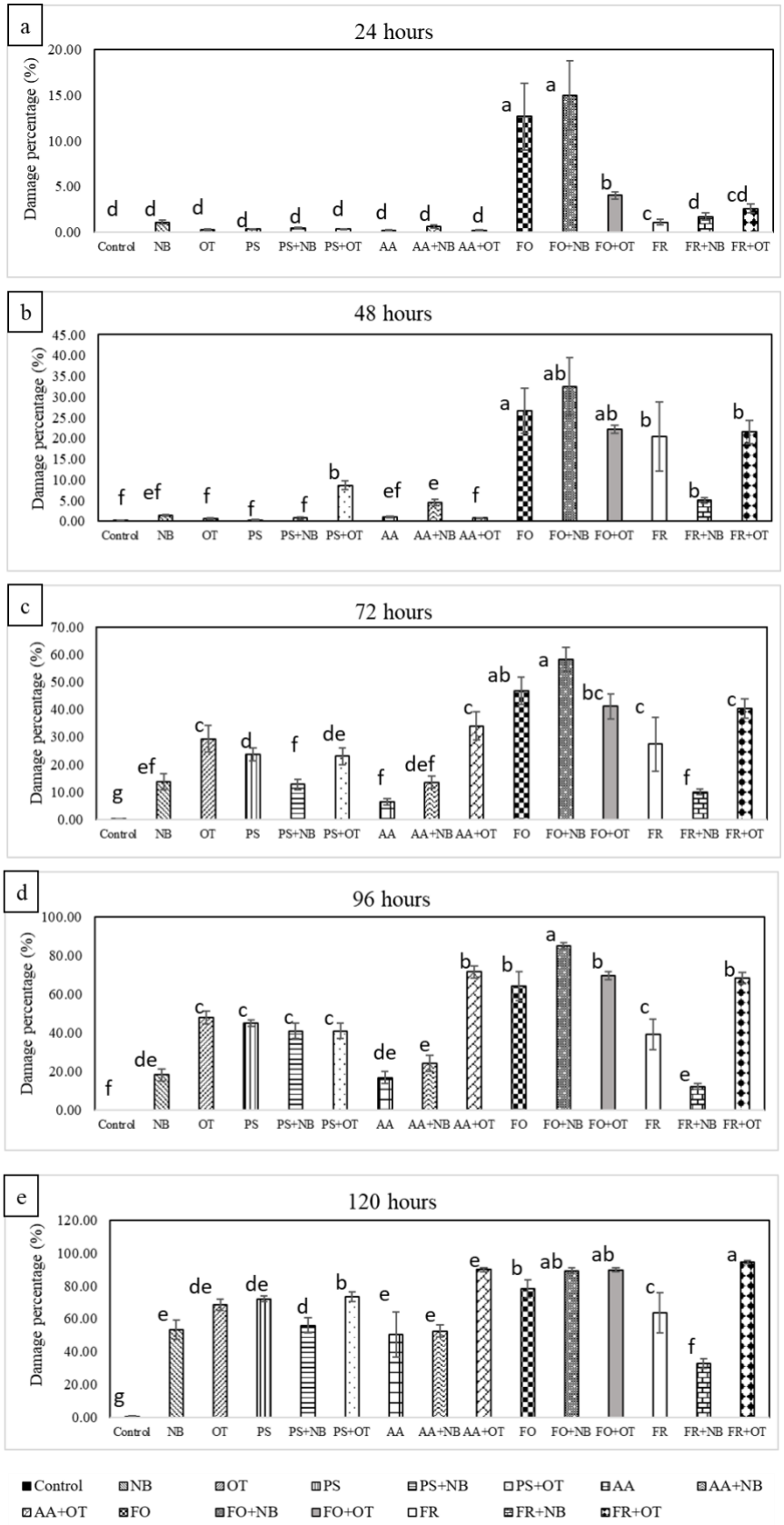


Figure 9: Arthropod-plant-pathogen interaction studies in various combinations at intervals of (a) 24hours; (b) 48 hours; (c) 72 hours; (d) 96 hours and (e) 120 hours respectively. Mean bars having different alphabets are significantly different ($P<0.05$)

Table 2: List of the phytopathogenic fungi isolated from waterhyacinth and their disease symptoms

Isolate No.	Fungal Isolate	Symptom of disease
WHK-17	*Acremonium* sp.	Zonate leaf spots
WHK-3#	*Alternaria alternata* (Fr.) Keissler	Leaf spots
WHK-34#	*Alternaria alternata* (Fr.) Keissler	Leaf spot and blight
WHK-38#	*Alternaria alternata* (Fr.) Keissler	Brownish leaf spots
WHK-42#	*Alternaria alternata* (Fr.) Keissler	Leaf spot and blight
WHK-43#	*Alternaria alternata* (Fr.) Keissler	Scattered leaf spots
WHK-141#	*Alternaria alternata* (Fr.) Keissler	Leaf spots
WHK-12#	*Alternaria japonica* Yoshii 1941	Leaf spots
WHK-7	*Alternaria* sp.	Leaf spots
WHK-46	*Alternaria* sp.	Leaf spots
WHK-104#	*Arthrinium hispanicum* Larrondo & Calvo	Leaf spots
WHK-89	*Cercospora* sp.	Leaf spots
WHK-10	*Cladosporium* sp.	No symptoms
WHK-21	*Cladosporium* sp.	No symptoms
WHK-24	*Cladosporium* sp.	No symptoms
WHK-103	*Cladosporium* sp	No symptoms
WHK-135	*Cladosporium* sp	No symptoms
WHK-1#	*Cochliobolus geniculatus* R.R. Nelson	Leaf blight and spot blotch
WHK-23#	*Cochliobolus geniculatus* R.R. Nelson	Leaf spot blotch
WHK-25	*Curvularia* sp.	Small necrotic spots
WHK-27	*Curvularia* sp.	Small necrotic spots
WHK-28	*Curvularia* sp.	Small necrotic spots
WHK-93	*Curvularia* sp.	Small necrotic spots
WHK-94	*Curvularia* sp.	Small necrotic spots
WHK-101	*Curvularia* sp.	Small necrotic spots
WHK-26	*Fusarium oxysporum (Schlecht)*	Irregular marginal spots
WHK-59#	*Fusarium oxysporum f.* sp. *lycopersici*	Leaf necrosis
WHK-106#	*Fusarium oxysporum f* sp. *cumini* strain F11	Leaf necrosis
WHK-71	*Fusarium roseum* Link	Leaf blight
WHK-9	*Fusarium ventricosum*	Zonate leaf spots
WHK-74#	*Fusarium verticillioides* (Sacc.) Nirenberg	Leaf spot and blight
WHK-87#	*Fusarium verticillioides* (Sacc.) Nirenberg	No symptom
WHK-13	*Fusarium* sp	Leaf spots
WHK-88	*Fusarium* sp	Central dark brown spots
WHK-92	*Fusarium* sp	Leaf spots
WHK-99	*Fusarium* sp	Leaf spots

WHK-136	*Fusarium* sp.	Leaf spots
WHK-30	*Myrothecium* sp.	Leaf lesions
WHK-142	*Nigrospora* sp.	Leaf blight
WHK-37[#]	*Paradendryphiella salina* (G.K. Sutherland) Woudenberg & Crous	Chlorotic patches
WHK-97[#]	*Pleospora herbarum* (Pers.) Rabenh.	Oval yellow spots
WHK-137[#]	*Pleospora herbarum* (Pers.) Rabenh.	Oval yellow spots
WHK-138[#]	*Pleospora herbarum* (Pers.) Rabenh.	Oval spots
WHK-20	*Rhizoctonia* sp.	Leaf blight and spots
WHK-96[#]	*Setosphaeria rostrata* K.J. Leonard	Leaf spots
WHK-152	*Stemphylium* sp.	Leaf spots
WHK-19	*Trichothecium* sp	No symptoms
WHK-36[#]	*Ulocladium botrytis* Preuss	Leaf spots

[#] Disease index of over 50% of the total area of application (after 120 hours of application)

Table 3: Repeated measures of ANOVA results for various isolates in in different time points (24, 48, 72, 96 and 120 hrs).

Tests of Within-Subjects Effects						
Measure: Disease Density						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Hrs	Sphericity Assumed	314785.288	4	78696.322	3404.267	.000
	Greenhouse-Geisser	314785.288	3.161	99581.685	3404.267	.000
Hrs * Isolates	Sphericity Assumed	80223.210	120	668.527	28.919	.000
	Greenhouse-Geisser	80223.210	94.832	845.948	28.919	.000

Table 4: Worldwide distribution record of phytopathogenic fungi isolated from waterhyacinth during our study

Fungal Isolate	Location of isolation (Sl no. From table 1)	* Countries from which the isolate has previously been reported from WH
***Acremonium* sp.**	1, 7, 14	Australia, India, Pakistan, Panama, Peru, South Africa, Sudan, USA
***Alternaria alternata* (Fr.) Keissler**	4, 13	Australia, Bangladesh, Egypt, Ethiopia, India, Mexico, South Africa
***Alternaria japonica* Yoshii 1941**	13	India
***Alternaria* sp.**	1, 7, 13	Mexico, Sri Lanka, South Africa, USA
***Arthrinium hispanicum* Larrondo & Calvo**	5	#First Report
***Cercospora* sp.**	8	Brazil, India, Mexico, Nigeria, South Africa, USA, Venezuela, Zambia
***Cladosporium* sp.**	1	Hong Kong, India, Nigeria, South Africa, USA
***Cochliobolus geniculatus* R.R. Nelson**	13, 14	#First Report
***Curvularia* sp.**	1, 4, 12, 13	India, Nepal, Sri Lanka, Sudan, USA
***Fusarium oxysporum* (Schlecht)**	1	Australia, India, South Africa
Fusarium oxysporum f*. sp. *lycopersici	1	#First Report
***Fusarium oxysporum f* sp. *cumini* strain F11**	13	#First Report

Fusarium roseum **Link**	5, 6	India, United States
Fusarium ventricosum	13	#First Report
Fusarium verticillioides **(Sacc.) Nirenberg**	13, 14	#First Report
Fusarium **sp.**	1, 5, 6, 8, 9, 13	India, Mexico, Nigeria, Peru, Sri Lanka, South Africa, Uganda
Myrothecium **sp.**	1	Burma, India, Indonesia, Malaysia, Mexico, Nigeria, Philippines, Sri Lanka, South Africa, Thailand, Uganda
Nigrospora **sp.**	1	India, Mexico, South Africa, USA
Paradendryphiella salina **(G.K. Sutherland) Woudenberg & Crous**	1	India
Pleospora herbarum **(Pers.) Rabenh.**	1, 9	India
Rhizoctonia **sp.**	14	India, Panama, Thailand, USA
Setosphaeria rostrata **K.J. Leonard**	4	#First Report
Stemphylium **sp.**	13	South Africa, USA
Trichothecium **sp**	14	India, South Africa, Sri Lanka
Ulocladium botrytis **Preuss**	1	Egypt, Ethiopia, South Africa

Table 5: Pathogenicity of the four fungi on waterhyacinth

Sl.No.	Pathogens	Treatment	Disease percentages {Mean (±SD)}		
			Day 1	**Day 3**	**Day 5**
1	***Alternaria alternata***	Scratched	16.67 (±2.89)	65.00 (±13.23)	83.33 (±15.28)
		Unscratched	4.67 (±2.52)	43.33 (±10.41)	73.33 (±15.28)
2	***Fusarium oxysporum***	Scratched	20.25 (±9.07)	61 (±25.06)	85 (±23.61)
		Unscratched	12 (±2.89)	45.67 (±14.58)	76.67 (±15.81)
3	***Fusarium verticilloides***	Scratched	12.33 (±9.29)	50.00 (±10.00)	66.67 (±15.28)
		Unscratched	2.67 (±2.08)	42.00 (±13.11)	66.67 (±5.77)
4	***Paradendryphiella salina***	Scratched	0.54 (±0.10)	23.6 (±10.41)	67 (±12.42)
		Unscratched	0.34 (±0.15)	23.20 (±5.75)	71.4 (±4.43)
5	**Control**	Scratched	0.00	0.00	0.00
		Unscratched	0.00	0.00	0.00

Table 6: Host range testing of selected fungal pathogens on various crops and weed hosts

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
1.	Aizoaceae	*Trianthema portulacastrum* L.[c] (Horse-purslane/ Pathar chata)	-	+	-	-	-	+	-	+
2.	Amaranthaceae	*Alternanthera philoxeroides* (Mart.) Griseb. [b c] (Alligator weed/ Pani-khutura)	-	-	-	-	+	+	-	-
3.		*Alternanthera brasiliana* (L.) Kuntze. [c] brazilian joyweed	-	-	-	-	-	+	-	-
4.		*Alternanthera pungens* H.B & K. [c] khaki weed	-	-	-	-	-	-	-	-
5.		*Alternanthera sessilis* L. [c] (Sessile joyweed/ Kantewali santhi)	-	-	-	-	-	+	-	+
6.		*Amaranthus viridis* L. [a] (Amaranth/ Chaulai)	-	+	-	-	-	-	-	+
7.		*Amaranthus tricolor* L. [c] *(Tricolor amaranth)*	-	-	-	-	-	-	-	-
8.		*Amaranthus spinosus* L. [c] (Spiny pigweed)	-	-	-	-	-	-	-	+
9.		*Digera arvensis* Forssk [c] *(False amaranth)*	-	-	-	-	-	-	-	-
10.		*Celosia argentea* L [c] *c (Cock's comb)*	-	-	-	-	-	-	-	-

11.	Apiaceae	*Centella asiatica* L. [c] (Asian pennywort/ Brahmi)	-	-	-	-	-	-	-	-
12.	Araceae	*Pistia stratiotes* L. [b c] (Water lettuce)	-	+	-	-	-		-	+
13.		*Colocasia esculenta* (L.) Schott [c] (Taro)	-	-	-	-	-	-	-	-
14.	Asteraceae	*Ageratum conyzoides* L. [c] Goat weed	-	-	-	-	-	-	-	-
15.		*Blumea lacera* DC [c] (False oxtongue/ Kukurbanda)	-	-	-	-	-	-	-	+
16.		*Cichorium intybus* L. [c] (Chickory/ Kasani)	-	-	-	-	-	-	-	-
17.		*Parthenium hysterophorus* L. [c] (Parthenium/ Gajar ghas)	-	-	-	-	-	-	-	-
18.		*Sonchus arvensis* L. [c] (Perennial sowthistle/ Bhatkataiya)	-	-	-	-	-	-	-	-
19.		*Tagetes erecta* L. [a] (Marigold/ Genda)	-	-	-	-	-	-	-	-
20.		*Tridax procumbens* L. [c] (Coat buttons/ Phulani)	-	-	-	-	-	-	-	+
21.		*Vernonia cinerea* [c] (Little ironweed)	-	-	-	-	-	-	-	-
22.	Asclepiadiaceae	*Calotropis gigantea* [c] (Giant milkweed)	-	-	-	-	-	-	-	-
23.	Azollaceae	*Azolla pinnata* Brown [bc] (Feathered mosquitofern)	-	-	-	-	-	-	-	-

24.	Boraginaceae	*Heliotropium indicum* L. [c] (Indian heliotrope)	-	-	-	-	-	-	-	-
25.	Brassicaceae	*Brassica campestris* L. *var sarson* [a] (Rai/ Sarson)	-	-	-	-	-	-	-	-
26.		*Raphanus sativus* L. [a] (Raddish/ Gajar)	-	-	-	-	-	-	-	-
27.		*B. oleracea* L. *var. botrytis* [a] (Cauliflower/ Phool gobhi)	-	-	-	-	-	-	-	-
28.		*B. oleracea* L. *var. capitata* [a] (Cabbage/ Bandha gobhi)	-	-	-	-	-	-	-	-
29.		*Sinapis alba* L. [c] (Wild mustard/ Safed Rai)	-	+	-	-	-	-	-	-
30.	Capparidaceae	*Cleome viscosa* L.[c] (asian spider flower)	-	-	-	-	-	-	-	-
31.	Caricaceae	*Carica papaya* L. [a] (papaya)	-	-	-	-	-	-	-	-
32.	Ceratophyllaceae	*Ceratophyllum demersum* L [b c] (hornwort, coontail)	-	-	-	-	-	-	-	+
33.	Chenopodiaceae	*Chenopodium album* L. [a c] (Goosefoot/ Bathua)	+	+	-	+	-	-	-	-
34.		*Spinacia oleracea* L. [a] (Spinach/ Palak)	-	+	-	+	-	-	-	+
35.	Commelinaceae	*Commelina benghalensis* L. [c] (Tropical Spiderwort/ Kanteri)	-	-	-	-	-	-	-	-
36.		*Cyanotis axillaris* (L.) D.Don ex Sweet [c] (Creeping Cradle Plant)	-	-	-	-	-	-	-	-

37.	Convolvulaceae	*Convolvulus arvensis* L. [c] (Bindweed/ Hiran chara)	-	-	-	-	-	-	-	-
38.		*Ipomoea fistulosa* Mart. [c] (Morning Glory/ Beshram)	-	-	-	-	-	-	-	-
39.		*Ipomoea aquatic* Forsk [b c] (Water spinach/ Kalmi sag)	-	+	-	-	-	-	-	-
40.	Cyperaceae	*Cyperus iria* L. [c] (Rice foot sedge/ Galmotha)	-	-	-	-	-	-	-	-
41.	Euphorbiaceae	*Euphorbia hirta* L. [c] (Asthma weed/ Dudhi)	-	-	-	-	-	-	-	-
42.	Fabaceae	*Cicer arietinum* L. [a] (Gram/ Chana)	-	-	-	-	-	-	-	-
43.		*Glycine max* L. [a] (Soybean)	-	-	-	-	-	-	-	-
44.		*Lens esculenta* Moench [a] (Lentil/ Masoor)	-	-	-	-	-	-	-	-
45.		*Medicago polymorpha* L. [c] (Medick)	-	-	-	-	-	-	-	-
46.		*Pisum sativum* L. [a] (Pea/ Matar)	-	-	-	-	-	-	-	-
47.		*Trifolium alexandrium* L. [c] (Egyptian clove/ Barseem)	-	-	-	-	-	-	-	-
48.		*Vigna radiata* L. [a] (Mung bean/ Moong)	-	-	-	-	-	-	-	-
49.	Gramineae	*Brachiaria mutica* (Forsk.) Stapf. [c] (Para grass)	-	-	-	-	-	-	-	-

50.		*Cynodon dactylon* L. [c] (Bermuda grass/ Dubh)	-	-	-	-	-	-	-	-
51.		*Oryza sativa* L. [a] (Paddy/ Dhan)	-	-	-	-	-	-	-	-
52.		*Triticum aestivum* L. [a] (Wheat/ Gehoon)	-	-	-	-	-	-	-	-
53.		*Zea mays* L. [a] (Maize, Corn/ Bhutta)	-	-	-	-	-	-	-	-
54.	Hydrocharitaceae	*Hydrilla verticillata* (L. f.) Royle [b c] (Hydrilla)	-	+	-	-	-	-	-	-
55.	Lamiaceae	*Hyptis suaveolens* L. Point. [c] (Pignut/ Wilayati tulsi)	-	-	-	-	-	-	-	-
56.	Lemnaceae	*Lemna minor* L. [b c] (Common duckweed)	-	-	-	-	-	-	-	-
57.	Linaceae	*Linum usitatissimum* L. [a] (Linseed/ Alsi)	-	-	-	-	-	-	-	-
58.	Malvaceae	*Sida acuta* Burm. f. [c] (Common wire weed/ Kareta)	-	-	-	-	-	-	-	-
59.		*Gossypium hirsutum* [a] (Cotton, non bt)	-	-	-	-	-	-	-	-
60.	Marsileaceae	*Marsilea minuta* L. [bc] (Pepperwort)	-	-	-	-	-	-	-	-
61.	Polygonaceae	*Rumex obtusifolius* L. [c] (Broad-leaved dock/ Jungli palak)	-	+	-	+	-	-	-	+
62.	Pontederiaceae	*Eichhornia crassipes* (Mart.) Solms (Waterhyacinth/ Jal kumbhi)	+	+	+	+	+	-	+	+

63.		*Monochoria vaginalis* [bc] (Pickerel weed)	-	-	-	-	-	-	-	-
64.	Solanaceae	*Lycopersicon esculentum* Mill. [a] (Tomato/ Tamaatar)	-	-	-	-	-	-	-	+
65.		*Physalis minima* L. [c] (Wild gooseberry/ Pachkotta)	-	-	-	-	-	-	-	-
66.	Trapaceae	*Trapa natans* L. [b] (Water chestnut)	-	-	-	-	-	-	-	-
67.	*Verbenaceae*	*Lantana camara L.* [c] (*Lantana*/ *Makoiya)*	-	-	-	-	-	-	-	-

[a] Cultivated plant

[b] Plant ecologically related to the test plant

[c] Non-native weed

[d] Plant reported susceptible to cultivars of *A. japonica*

* Spore suspension was sprayed in water containing hydrilla while phytotoxicity was accessed by growing the hydrilla shoot in the culture filtrate.

+ damage caused, - no damage

s- Spore suspension (5×10^5 spores/ml)

t- Culture filtrate

3. OBJECTIVE 3: *Studies on enzymes released from the selected fungal strains*

AA and FO from the previous objective have shown more efficacy in their disease-causing potential against the targeted waterhyacinth in comparison to the other two potent phytopathogens, PS and FR, with even a narrow host range specificity. Hence enzymes produced by AA and FO were analysed for their activity and biocontrol potential.

For AA, β –Glucosidase (70.6%±11.85) followed by endoxylanase (64.8%±9.43) and polygalactonurase or PGase (50%±10.65), have shown effective pathogenicity against waterhyacinth. However, the quantity of β –Glucosidase is much less than that of endoxylanase and PGase (Figure 10).

In case of FO, endoxylanase (81.8%±8.65) showed maximum biocontrol potential, followed by PGase (58%±6.44) and β –Glucosidase (57.6%±10.16), as evident from the disease index (Figure 11) against the host weed. However, endoxylanase have a much lesser activity in terms of PGase, its pathogenicity rate and the disease incurred by the enzyme was much higher.

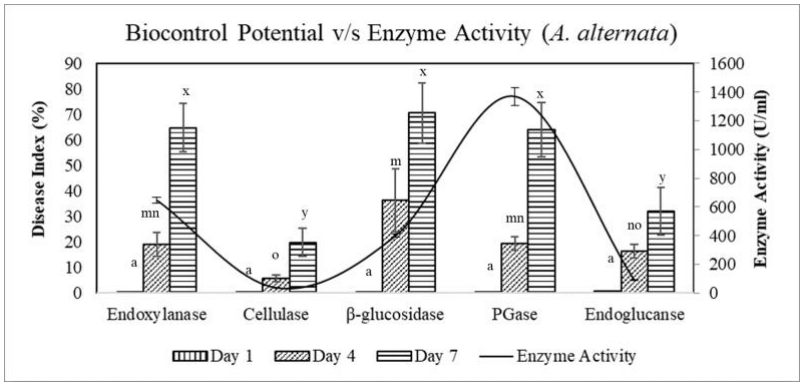


Figure 10: Pathogenicity and activities of various enzymes produced by *A. alternata* (Mean values having different superscripts are significantly different (P<0.05))

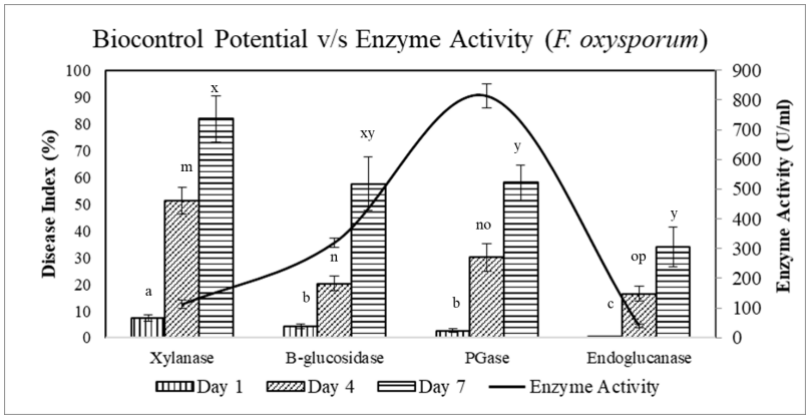


Figure 11: Pathogenicity and activities of various enzymes produced by *F. oxysporum* f. sp. *lycopersici* 4287 (Mean values having different superscripts are significantly different (P<0.05))

4. OBJECTIVE 4: *Biocontrol potential of the enzymes and their observation at tissue level*

Enzymes of FO and AA have both shown potential against the target weed, but due to high level of autotoxicity of the latter, maintenance of the fungal culture was difficult and hence only enzymes from FO were used to watch their potentiality and tissue level effect on waterhyacinth.

On optimizing the cultivation time, for the hemicellulase group of enzymes released from FO (Figure 12), the most favourable time for endoxylanase (EC 3.2.1.8) and endoglucanase (EC 3.2.1.4) was 72 hours and 96 hours respectively. Fungus failed to produce β–Glucosidase (EC 3.2.1.21). Polygalactonurase (EC 3.2.1.15) produced, was highly active after 96 hours. Endoxylanase and endoglucanase, from FO, were active at low temperature of 7^{o} C and even at 27^{o} C. When the activity of endoxylanase ceased after 27^{o}C, endoglucanase slowly rose with increase of temperature after 27^{o}C. Polygalactonurase was highly active at 27^{o} C. On varying the concentrations of different substrates, endoxylanase and polygalactonurase, both showed its optimum activity in growth media containing 1.5% of pure xylan and pectin, while endoglucanase using 0.5 % (w/v) of carboxy methyl cellulase (CMC). All the active enzymes produced showed their optimised working activity at a lower pH, favouring acidic growing condition.

Partially purified optimized endoxylanase followed by polygalactonurase and β–Glucosidase, from FO, have shown effective results against the weed, waterhyacinth, causing infections within a week of application (Figure 13). Hence, endoxylanase (or xylanase) was used for understanding the next level of enzymatic mechanism for cell wall degradation of the target weed, waterhyacinth, in microscopic level.

The microscopic image of transverse tissue section of waterhyacinth leaves shows slow degradation and dissolution of the cell layers (Figure 14, under 10X magnification) few hours after incubation with the xylanase, hinting at potency of the enzyme for cell wall degradation and biocontrol of the waterhyacinth. The epidermal layers of transverse section waterhyacinth leaf, shows a peeling off effect because of xylanase treatment (Figure 15, under 40X magnification).

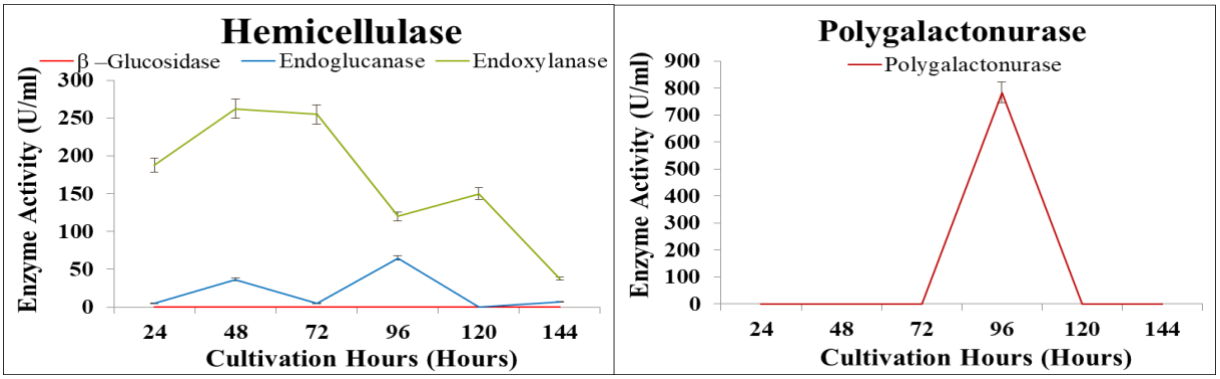
Confirmation of the presence of active genes of the potent fungal enzyme by expression profiling

The xylan-inoculated media grown fungal mat showed higher expression (for XYL2, XYL3 and XYL5, from FO) than no substrate induced growing media, with the former having a thicker band intensity difference than the later one (Figure 16), hinting at the activation of particular enzyme producing genes (XYL2, XYL3 and XYL5), in presence of xylan induced growing media.

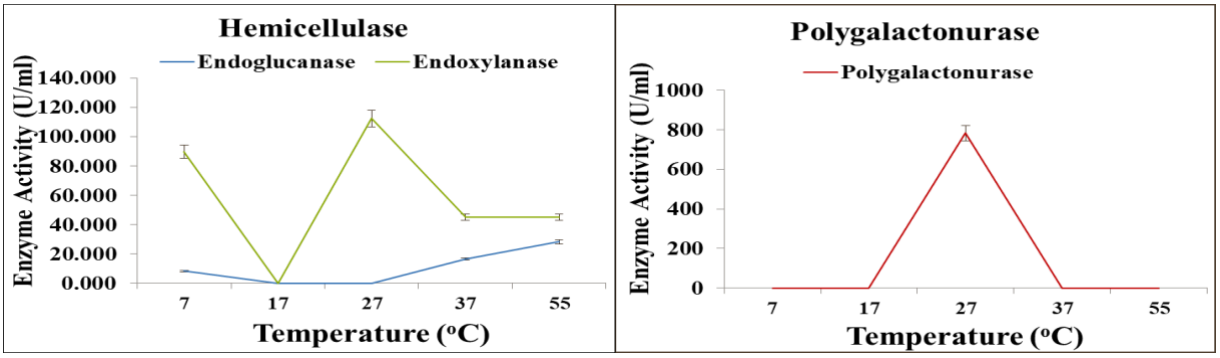
Phylogenetic analysis of the active genes of the potent fungal enzyme

The phylogenetic analysis of the XYL2, XYL3 and XYL5 genes of the xylanases of FO showed that it belonged to different clades with respect to the respective xylanase genes produced by other *Fusarium* sps (Figure 17).

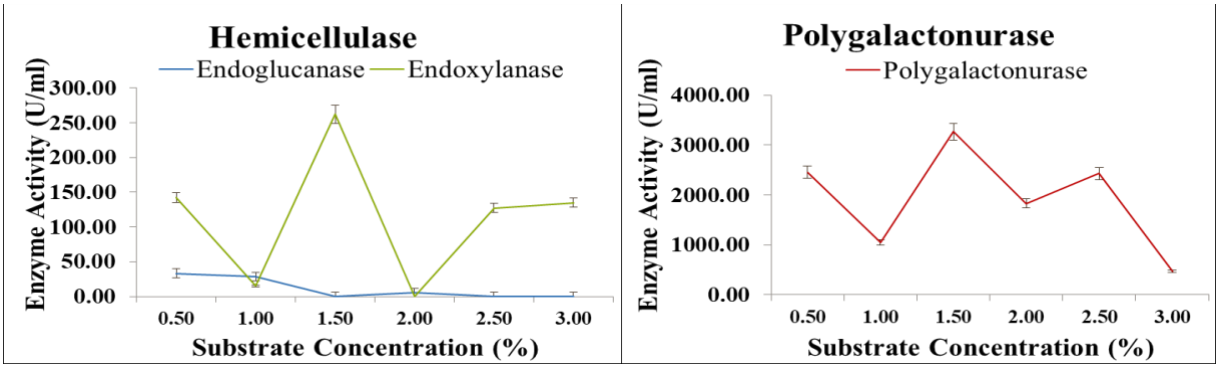
F. coffeatum, *F. venenatum*, *F. pseudograminearum* and *F. graminearum* (missing in case of XYL5), belong to an evolved monophyletic group with a significant number of deletions in the amino acid sequences. In comparison to the phyletic-outgroup (involving *F. proliferatum*, *F. fujikuroi* and *F. verticilloides*) they lie in the evolutionary pathway, of the xylanase genes, from *F. oxysporum* sps. to the initially mentioned monophyletic group.



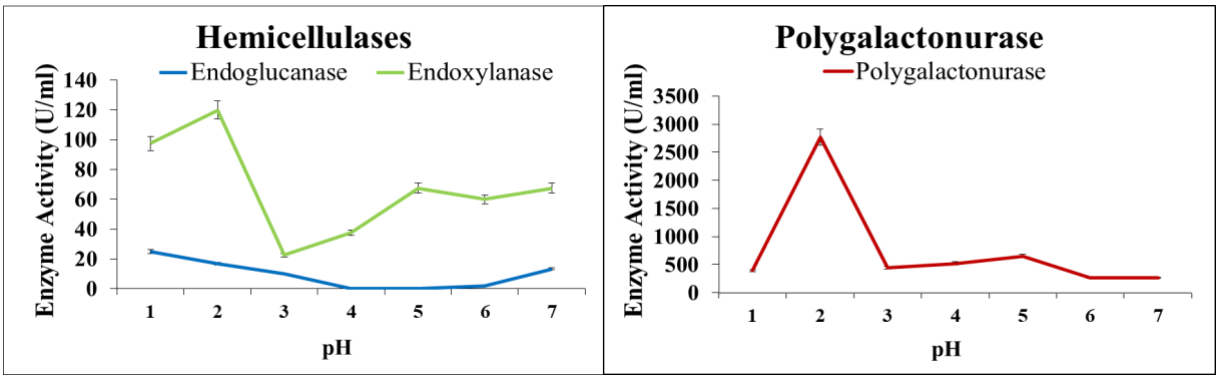
Optimisation of incubation time



Optimisation of Incubation temperature



Optimisation of substrate concentration



Optimisation of pH of the growing media

Figure 12: Studies on fungal enzymes from *Fusarium oxysporum* f. sp. *lycopersici* 4287

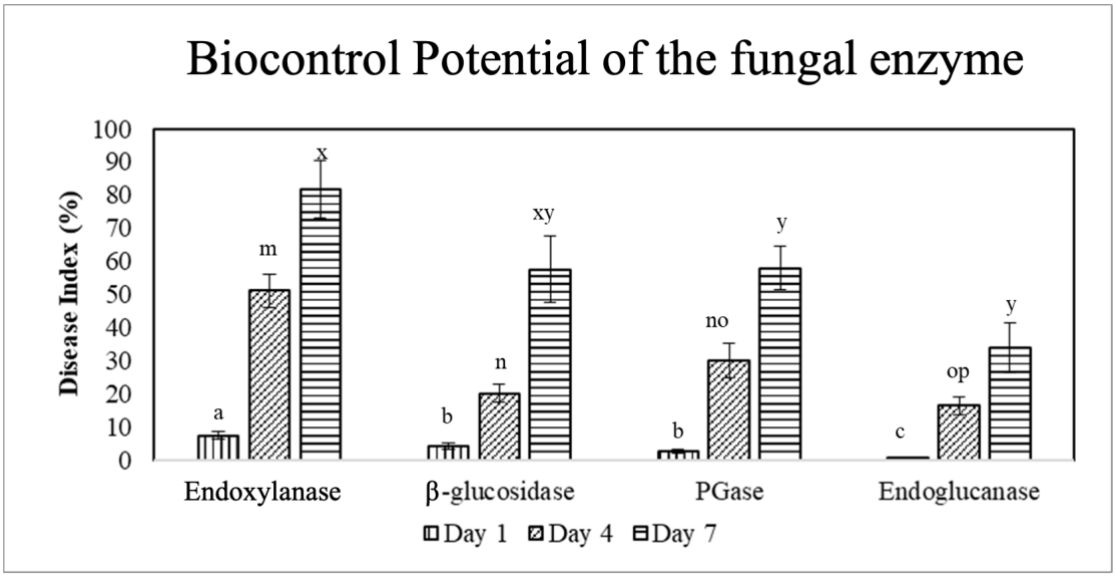


Figure 13: Pathogenicity test of the fungal enzymes (Mean values having different superscripts are significantly different (P<0.05))

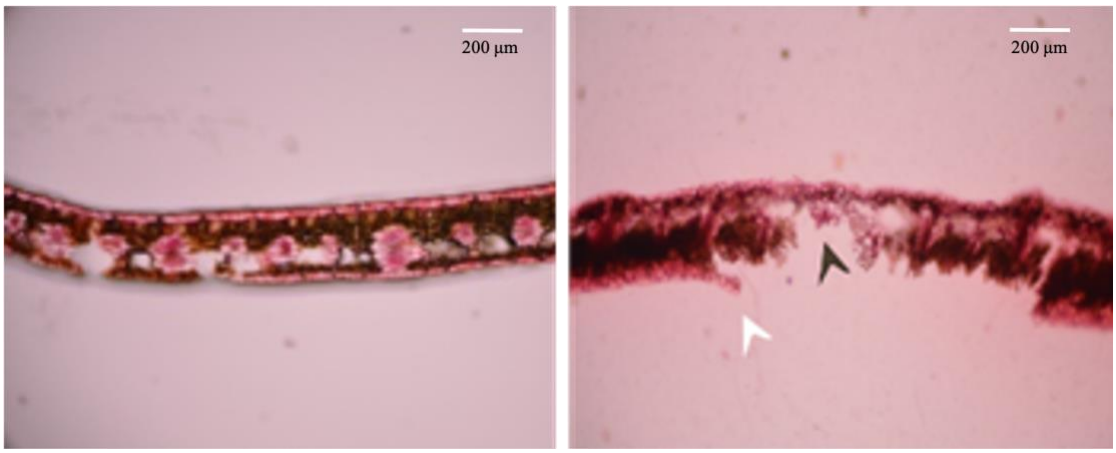


Figure 14: Slow dissolution and disintegration of the cell membranes, when the tissue was incubated with xylanase enzyme from *F. oxysporum* f. sp. *lycopersici* 4287

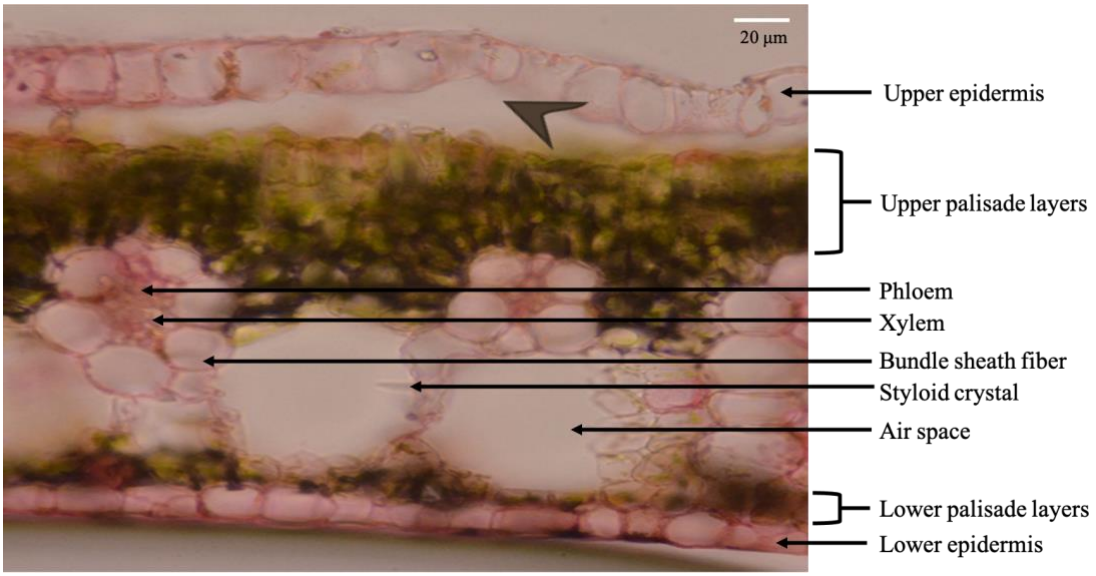


Figure 15: Peeling-off of the outer epidermal layer of the waterhyacinth leaf, because of effect of xylanase treatment

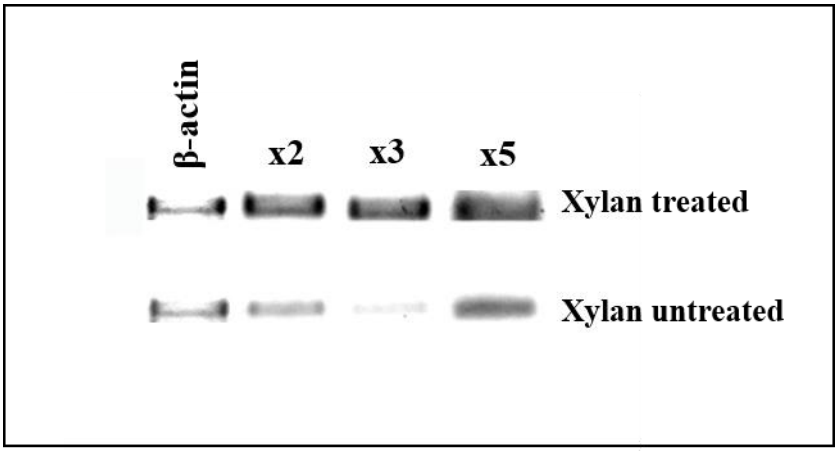
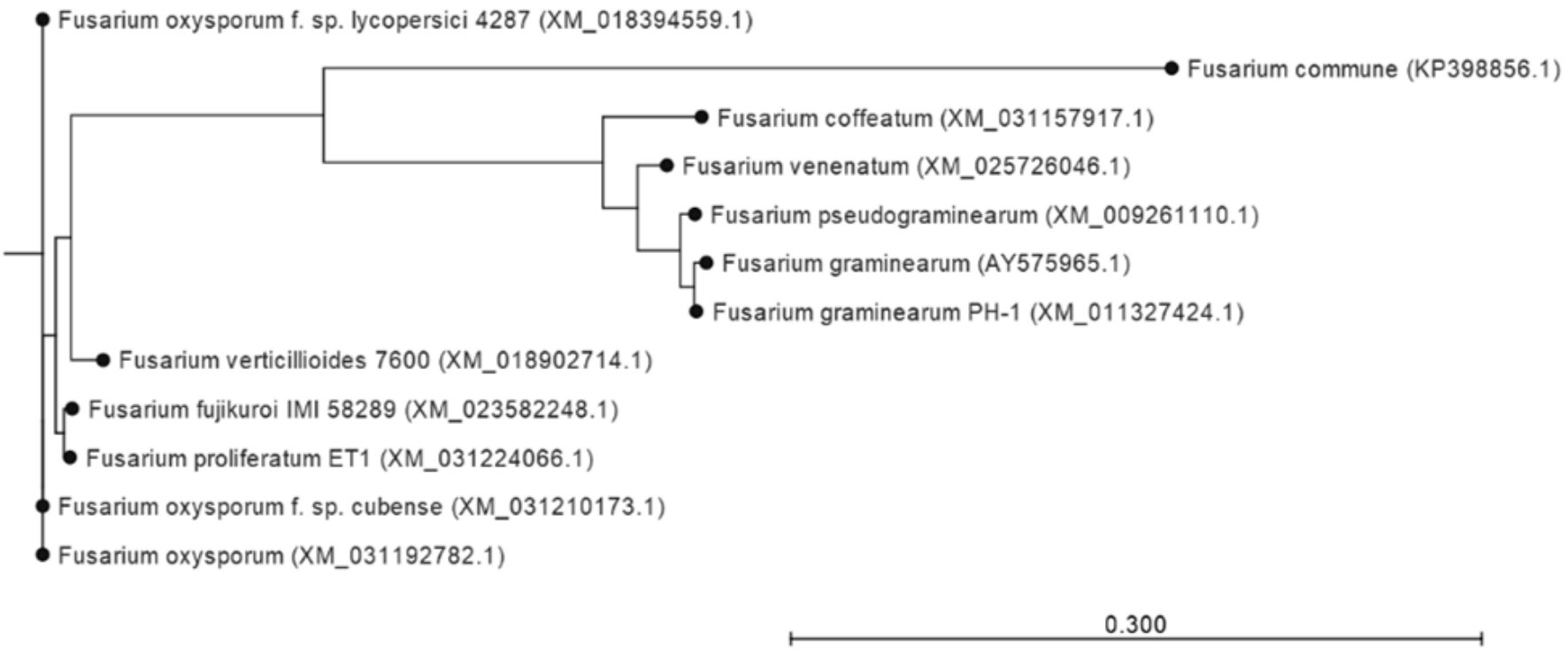
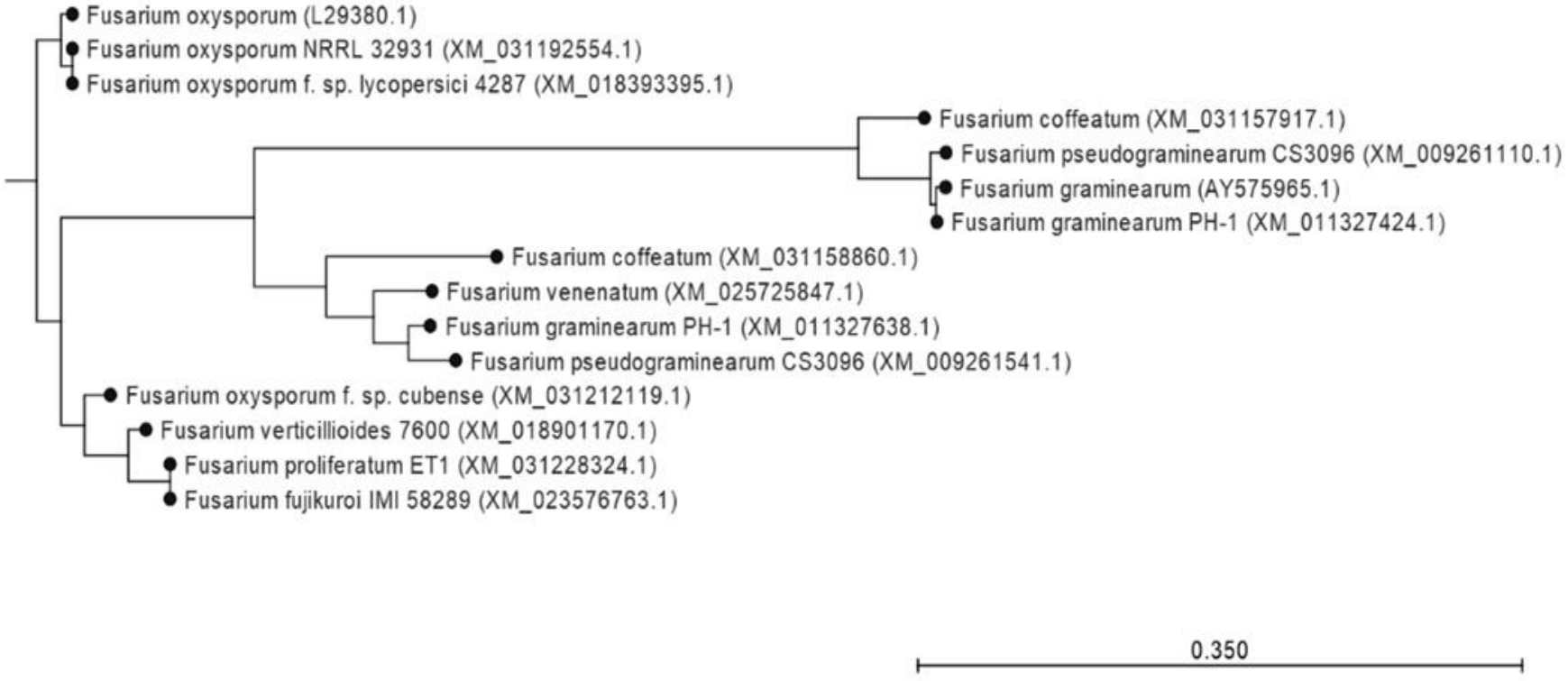


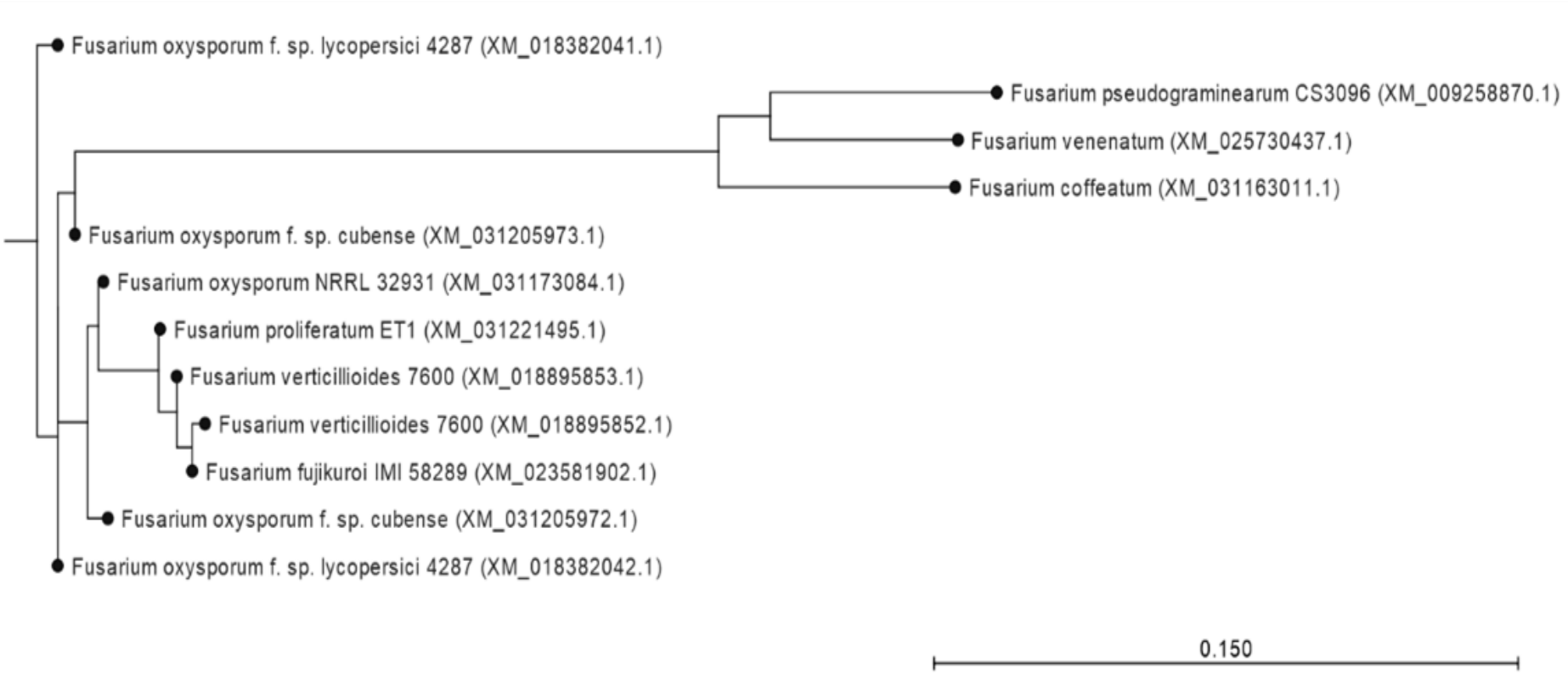
Figure 16: Amplification of the cDNA of xylanase family (XYL2, XYL3 and XYL5) enzyme, produced by *Fusarium oxysporum* f. sp. *lycopersici* 4287, observed on both treated and untreated xylan-inoculated growth media, with reference to β-actin (house-keeping gene) as control


Fusarium oxysporum f. sp. lycopersici 4287 (XM_018394559.1)
Fusarium commune (KP398856.1)
Fusarium coffeatum (XM_031157917.1)
Fusarium venenatum (XM_025726046.1)
Fusarium pseudograminearum (XM_009261110.1)
Fusarium graminearum (AY575965.1)
Fusarium graminearum PH-1 (XM_011327424.1)
Fusarium verticillioides 7600 (XM_018902714.1)
Fusarium fujikuroi IMI 58289 (XM_023582248.1)
Fusarium proliferatum ET1 (XM_031224066.1)
Fusarium oxysporum f. sp. cubense (XM_031210173.1)
Fusarium oxysporum (XM_031192782.1)
0.300

a. XYL 2


Fusarium oxysporum (L29380.1)
Fusarium oxysporum NRRL 32931 (XM_031192554.1)
Fusarium oxysporum f. sp. lycopersici 4287 (XM_018393395.1)
Fusarium coffeatum (XM_031157917.1)
Fusarium pseudograminearum CS3096 (XM_009261110.1)
Fusarium graminearum (AY575965.1)
Fusarium graminearum PH-1 (XM_011327424.1)
Fusarium coffeatum (XM_031158860.1)
Fusarium venenatum (XM_025725847.1)
Fusarium graminearum PH-1 (XM_011327638.1)
Fusarium pseudograminearum CS3096 (XM_009261541.1)
Fusarium oxysporum f. sp. cubense (XM_031212119.1)
Fusarium verticillioides 7600 (XM_018901170.1)
Fusarium proliferatum ET1 (XM_031228324.1)
Fusarium fujikuroi IMI 58289 (XM_023576763.1)
0.350

b. XYL3


Fusarium oxysporum f. sp. lycopersici 4287 (XM_018382041.1)
Fusarium pseudograminearum CS3096 (XM_009258870.1)
Fusarium venenatum (XM_025730437.1)
Fusarium coffeatum (XM_031163011.1)
Fusarium oxysporum f. sp. cubense (XM_031205973.1)
Fusarium oxysporum NRRL 32931 (XM_031173084.1)
Fusarium proliferatum ET1 (XM_031221495.1)
Fusarium verticillioides 7600 (XM_018895853.1)
Fusarium verticillioides 7600 (XM_018895852.1)
Fusarium fujikuroi IMI 58289 (XM_023581902.1)
Fusarium oxysporum f. sp. cubense (XM_031205972.1)
Fusarium oxysporum f. sp. lycopersici 4287 (XM_018382042.1)
0.150

c. XYL 5

Figure 17: The phylogenetic (neighbour-joining) tree of *F. oxysporum* sp. *lycopersici* 4287 with respect to the other closely related *Fusarium* sps. based on the xylanase (a. XYL2; b. XYL3; c. XYL5) genes. The horizontal branches are proportional to the number of the amino acid substitutions per site.

VI. DISCUSSION

Periodical surveys have highlighted the fact that a rich diversity of fungal pathogens is associated with waterhyacinth in and around Kolkata, India, where it was first introduced in the country. Many of these fungal pathogens possibly share a common association with those documented earlier from the Amazon River Basin, the place of origin of the weed, (Ray and Hill, 2013; Evans and Reeder 2001) and other countries invaded by waterhyacinth (Ray and Hill, 2013; Firehun et al. 2017). Several pathogens possibly co-evolved with waterhyacinth and were spread to different parts of the globe with the weed itself. But the pathogenicity of these fungi even varies from one isolate to another as recorded in case of *F. oxysporum* by Praveena and Naseema (2006). Among the 152 pathogens collected, isolates of *A. alternata, A. japonica, F. oxysporum, F. verticilloides, U. botrytis* and *P. salina* appeared the most potent with narrow host-range and better adapted for cell wall degradation of the monocot (King et al. 2011). Apart from the reports of some effective ancient mycobiota from the plants native range (i.e., *A. alternata*, *F. oxysporum*) reports of freshly emerging adaptive pathogens, like *A. japonica, Diplodia mutila*, from the introduced range of the weeds are also playing a role in controlling the weed (Dutta et al 2015, Singh et al 2016).

Certain fungal pathogens enzymatically detoxify the saponins present in the secondary metabolite produced by plants, because they form complexes with the membrane sterols, leading to disintegration of the membrane of plant, and on such interaction, infection susceptibility the host range specificity of the fungi is dependent (Bowyer et al. 1995).

Host specific metabolites are unique and are effective only to plants that are susceptible to the fungi that produce those (Babu et al 2003). No significant correlation was drawn where the pathogenicity of the enzymes could be attributed to the enzyme activity (Schäfer 1994). The fungal enzymes' activity from *F. oxysporum*, when grown on various media showed their best activity in Rose Bengal Chloramphenicol. The influence of culture media may be a significant factor in the production of a highly effective inoculum needed for control of waterhyacinth (Babu et al 2003). Polygalactonurase of *F. oxysporum* showed optimum temperature activity of 27^{o}C like *Aspergillus* sp (Galiotou-Panayotou et al 1997). Polygalactonurase showed maximum production within 96 hours after which exhaustion of nutrients in the medium resulted in drop of activity. Endoglucanase, endoxylanase activity is usually, among many fungi, after 80 hours and 48 hours of inoculation (Ray et al 2013a,b). When endoxylanase works best at lower temperature, β–Glucosidase can work at higher temperature, though the later was not produced by *F. oxysporum* (Ray et al 2013b). All enzyme produced are usually restricted using lower substrate concentration cause increase in substrate concentration result in drastic reduction in enzyme production probably due to catabolic repression of biosynthesis (Omojasola et al. 2008) or as a result of reduced mass transfer of oxygen by higher amount of solid substrate (Ghosh and Ray 2011). The amount of mycelium present was directly responsible for the quantity of uptake of cations and anions and efflux of ammonia (Xue et al. 2018), which affected the pH, hence a lower

pH with large number of ions is preferable for the enzymes to work efficiently and actively.

The presence of CWDEs has been reported in healthy tissue in several plants (Jayasinghe et al. 2004, Chaurasia et al. 2014). Similar to the trend, traces of CWDE activity were detected in healthy tissues in our study (as the control sets of treatment did show pathogenicity effects when applied on waterhyacinth), but we cannot exclude that this trace activity was due to the experimental conditions, for example, the use of a clear plastic bag to maintain high humidity for plant growth. Although plants activate the immune system when they encounter pathogenic microorganisms, including secreting enzyme (Shafikova and Omelichkina 2015) for the current study the plant tissues were necrotic due to infection by the pathogen. Therefore, we suggest that it is very likely that the major contribution to the overall quantity of the CWDEs present in the diseased tissue extracts is of fungal origin.

Alternaria have been reported earlier too for their autotoxic nature (Saxena and Pandey, 2009) and for that reason, and the survival of the fungi became so hectic that enzymes of *F. oxysporum* was chosen only for working to understand their biocontrol potential. The latter fungi have three xylanase genes belonging to the glycoside hydrolases family of the CAZy (Carbohydrate-active enzyme) database with a molecular weight around 22kDa. They either alone or in combination are required for the xylan growth production by the fungi (Apel-Birkhold and Walton 1996). Reports of β-xylosidase degrading minor sugars (Holden and Walton 1992) could also add to breakage of the cell wall moiety of the target host weed, thus enhancing the infection process. The presence of redundant enzymes is becoming common in many CWDEs and the significance of redundancy might be due to dissimilarity in their pattern of expression and/or activity. The Xyl2, Xyl3 and Xyl5 needs to be purified to assure the rest. However, the encoding genes have distinct patterns of expression. From a study of the cutinases and esterases of *Alternaria brassicicola*, Yao and Köller (1995) have drawn a distinction between enzymes typical of saprophytic growth and those associated with pathogenic growth. Phylogenetic analysis of the xylanase gene, related to the RNA expression profiling, were done to understand the related taxa and subgroups for the enzyme profiles to evolutionary lineages. Xylanses from *F. oxysporum* was found to be more closely related than the monophyletic clade to which the *F. coffeatum*, *F. venenatum*, *F. pseudograminearum* and *F. graminearum* belongs. Though phylogenetic evidence depending on a particular gene is very insufficient to justify taxonomic evolutionary lineages (Rokas et al. 2003), but works on the toxigenic potential and phylogenetic correlation in *Fusarium* sps. have been undertaken lately too (Kristensen et al. 2004).

VII. CONCLUSION

In the world of increasing development and application of xenobiotic compounds, such as chemical pesticides, using eco-friendly biological control methods becomes absolute

necessity to control aquatic weeds without bioaccumulation or leaving a negative impact on the surrounding ecosystem. Using metabolites produced by certain phytopathogenic fungi would be more effective, host-specific and eco-friendly (Saxena, and Pandey, 2001). With varying environmental conditions in tropical and subtropical regions of the world, where waterhyacinth has invaded, diversity of biological control agents have fluctuated with change in varying conditions (Praveena and Naseema, 2006). So, proper understanding and likewise application and improving biocontrol strategies by developing effective mycoherbicides is essential for improving biocontrol mechanism to work in field conditions.

VIII. FUTURE DIRECTIONS AND SIGNIFICANCE

Unlike secondary metabolites produced by selected fungi, all produce primary metabolites. Several reports have suggested the use of the former in weed-biocontrol. The thesis highlights the use of primary metabolites, in cell wall degradation of the host weed and disintegrating it, playing a pivotal role in its management. The study also redirects to the involvement of the particular gene that play a pivotal role in the degradation process.

The study broadens the use of more specific eco-friendly means of controlling the aquatic weed, waterhyacinth and at much faster rates by using the primary out products of the fungi, those released during the initial period of the growth phase of the microbe.

IX. ACKNOWLEDGEMENT

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XI. Plagiarism Certificate

URKUND

Document Information

Analyzed document	Writuparna Dutta's-PhD 5000- for Plagiarism Check.docx (D88143154)
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W	URL: https://www.researchgate.net/publication/233987786_Survey_and_selection_of_potenti ... Fetched: 7/7/2020 8:35:26 AM	2
J	**Fungi associated with Eichhornia crassipes in South Africa and their pathogenicity under controlled conditions** URL: dc64377a-e8b8-4363-8504-c40030ed7083 Fetched: 3/13/2019 4:58:50 AM	6
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W	URL: https://edepot.wur.nl/401611 Fetched: 10/10/2020 8:28:45 AM	1

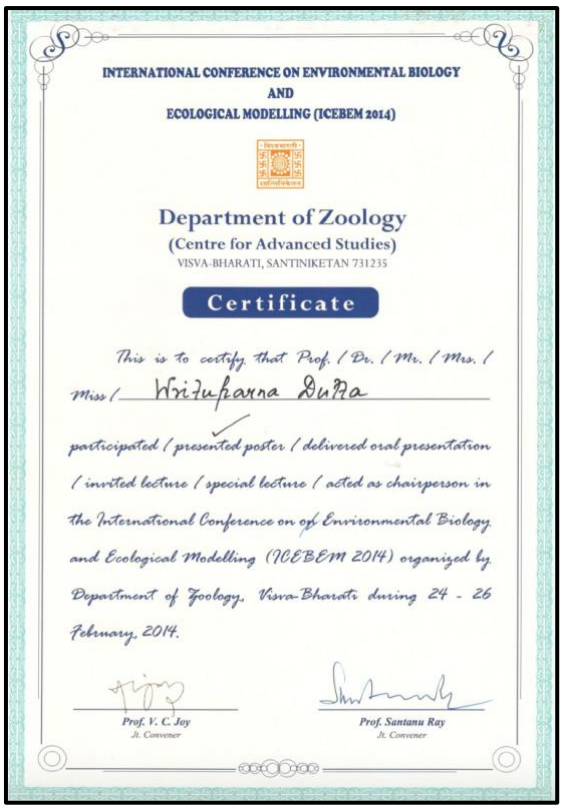
Writuparna Dutta

Puja Ray

XII. Additional Documents:

a. Certificate of conferences attended (some selected ones)

i. 2014



INTERNATIONAL CONFERENCE ON ENVIRONMENTAL BIOLOGY
AND
ECOLOGICAL MODELLING (ICEBEM 2014)

Department of Zoology
(Centre for Advanced Studies)
VISVA-BHARATI, SANTINIKETAN 731235

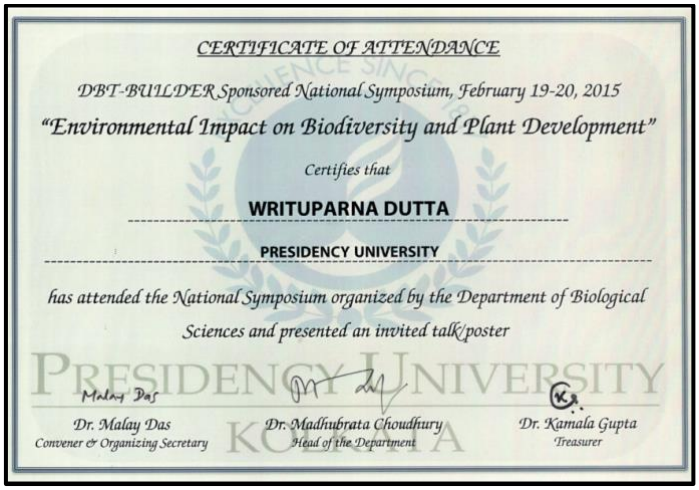
Certificate

This is to certify that Prof. / Dr. / Mr. / Mrs. / Miss / Writuparna Dutta ✓ participated / presented poster / delivered oral presentation / invited lecture / special lecture / acted as chairperson in the International Conference on of Environmental Biology and Ecological Modelling (ICEBEM 2014) organized by Department of Zoology, Visva-Bharati during 24 - 26 February, 2014.

Prof. V. C. Joy
Jt. Convener

Prof. Santanu Ray
Jt. Convener

ii. 2015



CERTIFICATE OF ATTENDANCE

DBT-BUILDER Sponsored National Symposium, February 19-20, 2015

"Environmental Impact on Biodiversity and Plant Development"

Certifies that

WRITUPARNA DUTTA

PRESIDENCY UNIVERSITY

has attended the National Symposium organized by the Department of Biological Sciences and presented an invited talk/poster

Malay Das

Dr. Malay Das
Convener & Organizing Secretary

Dr. Madhubrata Choudhury
Head of the Department

Dr. Kamala Gupta
Treasurer

PRESIDENCY UNIVERSITY KOLKATA

iii. 2016

Certificate of Attendance

We hereby certify that

Dutta Writuparna

has attended the

7th International Weed Science Congress

held at the Clarion Congress hotel Prague, Czech Republic
June 19–25, 2016.

7th International Weed Science Congress
Prague, Czech Republic

IWSS

WSSA

Writuparna Dutta

Winner of the IWSS Travel Award

in Recognition of an Outstanding Proposed Presentation
for the 7th International Weed Science Congress
held in Prague, Czech Republic, 19-25 June 2016

Nilda Roma-Burgos
Acting President, IWSS

Franck Dayan
Treasurer, IWSS

iv. 2017

P57

The 26th Asian-Pacific Weed Science Society Conference

APWSS 2017 Kyoto, JAPAN

The Golden Jubilee Conference

Weed Science for People, Agriculture, and Nature

Celebrating 50 Years!

Certificate of Presentation

Writuparna Dutta

presented "**Evaluation of fungi, *Paradendryphiella salina* as potential biocontrol agent of aquatic weed, waterhyacinth**" as a speaker in the 26th Asian-Pacific Weed Science Society Conference (APWSS2017) , held between September 19th-22nd, 2017 in Kyoto, Japan.

Hiroshi Matsumoto
Chairperson, the 26th Asian-Pacific Weed Science Society Conference (APWSS2017)
President, Asian-Pacific Weed Science Society
Professor, University of Tsukuba

The 26th Asian-Pacific Weed Science Society Conference

APWSS 2017 Kyoto, JAPAN

The Golden Jubilee Conference

Weed Science for People, Agriculture, and Nature

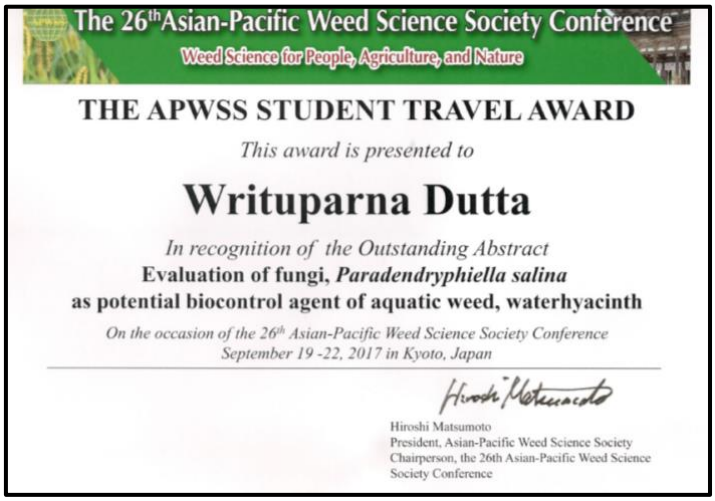
Celebrating 50 Years!

Certificate of Presentation

Writuparna Dutta

presented "**Studies on understanding the trends of invasive aquatic plant succession through inter-specific interactions among weeds**" as a speaker in the 26th Asian-Pacific Weed Science Society Conference (APWSS2017) , held between September 19th-22nd, 2017 in Kyoto, Japan.

Hiroshi Matsumoto
Chairperson, the 26th Asian-Pacific Weed Science Society Conference (APWSS2017)
President, Asian-Pacific Weed Science Society
Professor, University of Tsukuba



The 26th Asian-Pacific Weed Science Society Conference

Weed Science for People, Agriculture, and Nature

THE APWSS STUDENT TRAVEL AWARD

This award is presented to

Writuparna Dutta

In recognition of the Outstanding Abstract
Evaluation of fungi, *Paradendryphiella salina*
as potential biocontrol agent of aquatic weed, waterhyacinth

On the occasion of the 26th Asian-Pacific Weed Science Society Conference
September 19 -22, 2017 in Kyoto, Japan

Hiroshi Matsumoto
President, Asian-Pacific Weed Science Society
Chairperson, the 26th Asian-Pacific Weed Science Society Conference

v. 2018

ISWS Golden Jubilee
International Conference

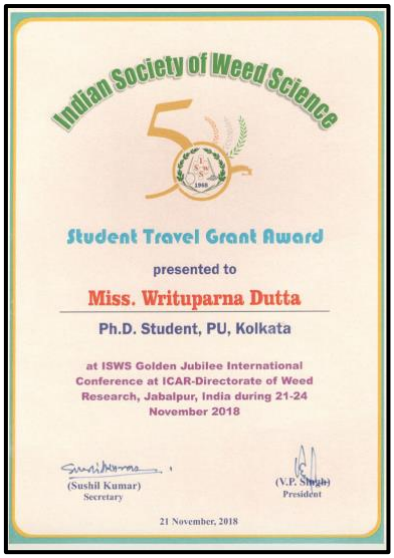
"Weeds and Society: Challenges and Opportunities"

Certificate of Participation

This is to certify that Miss. Writuparna Dutta
from Presidency University, Kolkata, West Bengal
participated and made an oral presentation in the ISWS Golden Jubilee International Conference held at ICAR-Directorate of Weed Research in Jabalpur, India during 21-24 November, 2018.

(Sushil Kumar)
Organizing Secretary

(V. Pratap Singh)
President, ISWS



Indian Society of Weed Science

Student Travel Grant Award

presented to

Miss. Writuparna Dutta

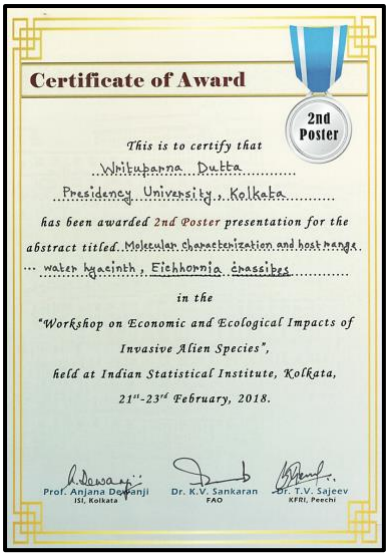
Ph.D. Student, PU, Kolkata

at ISWS Golden Jubilee International Conference at ICAR-Directorate of Weed Research, Jabalpur, India during 21-24 November 2018

(Sushil Kumar)
Secretary

(V.P. Singh)
President

21 November, 2018



Certificate of Award

2nd Poster

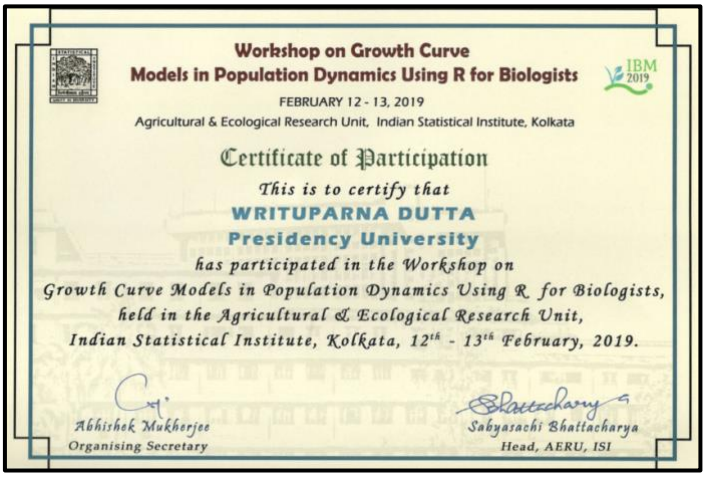
This is to certify that
Writuparna Dutta
Presidency University, Kolkata
has been awarded 2nd Poster presentation for the abstract titled Molecular characterization and host range ... water hyacinth, Eichhornia crassipes
in the
"Workshop on Economic and Ecological Impacts of Invasive Alien Species",
held at Indian Statistical Institute, Kolkata,
21st-23rd February, 2018.

Prof. Anjana Dewanji
ISI, Kolkata

Dr. K.V. Sankaran
FAO

Dr. T.V. Sajeev
KFRI, Peechi

vi. 2019



Workshop on Growth Curve
Models in Population Dynamics Using R for Biologists

IBM 2019

FEBRUARY 12 - 13, 2019
Agricultural & Ecological Research Unit, Indian Statistical Institute, Kolkata

Certificate of Participation

This is to certify that
WRITUPARNA DUTTA
Presidency University
has participated in the Workshop on
Growth Curve Models in Population Dynamics Using R for Biologists,
held in the Agricultural & Ecological Research Unit,
Indian Statistical Institute, Kolkata, 12th - 13th February, 2019.

Abhishek Mukherjee
Organising Secretary

Sabyasachi Bhattacharya
Head, AERU, ISI

vii. 2020

THE INDIAN SCIENCE CONGRESS ASSOCIATION

(Professional Body under Department of Science & Technology, Ministry of Science & Technology, Government of India)

14, Dr. Biresh Guha Street, Kolkata – 700 017

Participation Certificate

This is to certify that Prof./Dr./Shri/Smt. Writuparna Dutta of Presidency University, Kolkata has participated in the 107th Indian Science Congress held at University of Agricultural Sciences, Bangalore from January 3 to 7, 2020.

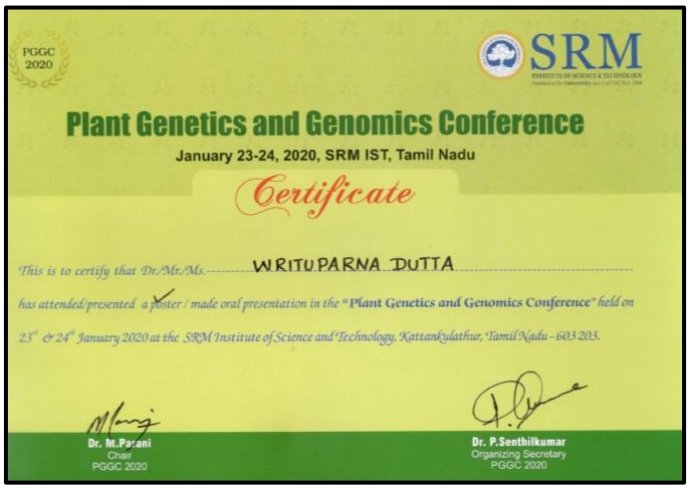
His/Her Membership Number is L30745

Date 7th January 2020

Dr. S. Ramakrishna
General Secretary
(Membership Affairs)

Office Seal

Dr. Anoop Kumar Jain
General Secretary
(Scientific Activities)



PGGC 2020

SRM

Plant Genetics and Genomics Conference

January 23-24, 2020, SRM IST, Tamil Nadu

Certificate

This is to certify that Dr./Mr./Ms. WRITUPARNA DUTTA has attended/presented a poster / made oral presentation in the "Plant Genetics and Genomics Conference" held on 23rd & 24th January 2020 at the SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu - 603 203.

Dr. M.Pa:ani
Chair
PGGC 2020

Dr. P.Senthilkumar
Organizing Secretary
PGGC 2020

b. First page of published manuscript (Selected ones: related to thesis)

Indian Journal of Weed Science **47**(1): 68–74, 2015

Molecular characterization and host range studies of indigenous fungus as prospective mycoherbicidal agent of waterhyacinth

Writuparna Dutta Durga Ray[1] and Puja Ray

Department of Biological Sciences, Presidency University, 86/1 College Street, Kolkata 700 073

Received: 3 January 2015; Revised: 8 March 2015

ABSTRACT

An indigenous fungal culture, isolated from diseased waterhyacinth, in Bolpur, Santiniketan, West Bengal, India, was found to be causing severe blight and dieback disease on waterhyacinth, under laboratory and field conditions. It was subjected to morphological and molecular characterization by amplification of 18S RNA gene fragment from genomic DNA using 18S gene universal primers. Subsequently with sequencing, GenBank database comparisons and phylogenetic analysis, the fungus was determined as *Alternaria japonica* Yoshii. Further we evaluated the pathogen for its host specificity to be developed as mycoherbicidal agent against this invasive weed. Host range of *A. japonica* was screened against 48 plant species in 42 genera representing 22 families in pot experiment. Waterhyacinth was the only species strongly susceptible to spore suspension (5×10^5 conidia/ml) of *A. japonica*. Minor infection was observed on goosefoot which is not only a weed but also ecologically separated from waterhyacinth. Thus the use of this pathogen in the biological control of waterhyacinth would be safe for plants of economic and ecological significance in India. We further sprayed the secondary metabolite produced by *A. japonica* on the test plants but phytotoxic symptoms were produced on nine out of 48 plants tested, demonstrating that phytotoxin produced by the fungus is not host specific. Further field tests needs to ascertain its efficacy under more natural conditions.

Key words: 18s RNA, *Alternaria japonica* Yoshii, Biological control, Host specificity, Mycoherbicide, Waterhyacinth

Phytoparasitica (2017) 45:429–437
DOI 10.1007/s12600-017-0605-y

A glimpse into the compatibilities and conflicts between arthropods and fungal biological control agents of aquatic weed waterhyacinth

Writuparna Dutta · Puja Ray

Received: 16 February 2017 / Accepted: 23 July 2017 / Published online: 4 August 2017
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Abstract Interspecific interactions play an important role in ecosystem functioning. Several biocontrol agents are being introduced for the control of invasive species like the aquatic macrophyte, waterhyacinth, *Eichhornia crassipes*, in the hope of improving the eco-friendly natural control mechanism. Due to their overlapping niches, interspecific interactions among the biocontrol agents is high and there is a paucity of information on possible positive or negative impact they may have on each other hence affecting the biocontrol. Therefore studies on their interaction become even more important. In order to understand such possibilities, three potential fungi, *Fusarium oxysporum* (FO), *F. roseum* (FR), *Paradendryphiella salina* (PS) and two host-specific arthropods *Orthogalumna terebrantis* (OT) and *Neochetina bruchi* (NB) were applied on water hyacinth plants singly and in various combinations (OT, NB, FO, FO + OT, FO + NB, FR, FR + OT, FR + NB, PS, PS + OT, PS + NB). Observations were made on percentage damage caused by these agents to water hyacinth. Biocontrol agents applied in combination had cumulative impact on the target weed. FO + NB and FO + OT treated plants showed enhanced damage percentage of about 90%, after 120 h. But FR + NB and PS + NB treated plants were far less damaged compared to when these agents were applied separately. PS also had a repelling effect on NB. Results of such interactions are often variable and less predictable, especially under field conditions. These results demonstrate the need for studies on biotic interactions among biocontrol agents, prior to release of the biocontrol agents of weeds.

Keywords Multitropic-interaction · Invasive species · Phytopathogen · Repulsive effect, weed biocontrol

Introduction

Waterhyacinth, *Eichhornia crassipes* Mart. Solms-Laubach (Pontederiaceae), a native of the tropical South America, is considered to be one of the most serious aquatic weeds (Holm et al. 1977). The climatic conditions of tropical and sub-tropical countries have favoured the invasiveness of the weed, causing wide-

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